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LE SUCRE RARE TAGATOSE: UN FONGICIDE ÉCOLOGIQUE CONTRE LES ESPÈCES DE *PHYTOPHTHORA*

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LE SUCRE RARE TAGATOSE: UN FONGICIDE ÉCOLOGIQUE CONTRE LES ESPÈCES DE *PHYTOPHTHORA*

Les espèces de *Phytophthora* comptent parmi les phytopathogènes les plus agressifs et les plus répandus dans le monde et causent d'énormes pertes économiques et dont le contrôle est principalement basé sur l'utilisation de produits chimiques. Cependant, l'utilisation excessive de pesticides chimiques contre les espèces de *Phytophthora* a fait apparaître de nombreuses menaces pour la santé humaine et l'environnement et de nombreux produits chimiques ont ainsi été bannis. L'objectif de cette thèse est d'étudier l'efficacité et le mécanisme d'action du sucre rare, le tagatose, contre les espèces de *Phytophthora* afin de permettre le développement de produits écologiques basés sur cet ingrédient actif. Le tagatose a inhibé la croissance mycélienne et a affecté négativement les processus mitochondriaux chez *P. infestans*, mais pas chez *P. cinnamomi*, indiquant des réponses différentes entre les deux espèces de *Phytophthora* vis à vis de ce sucre rare. L'inhibition de la croissance de *P. infestans* peut être attribuée aux similitudes structurelles du tagatose avec le fructose et à l'inhibition compétitive des enzymes du métabolisme des glucides. D'autre part, *P. cinnamomi* a été capable d'activer de multiples voies d'adaptation en activant les gènes impliqués dans le transport du glucose, le métabolisme des pentoses, le cycle de l'acide tricarboxylique, la détoxification des espèces réactives de l'oxygène et la respiration mitochondriale et alternative. En outre, le tagatose affecte la composition des bactéries de la phyllosphère et augmente le nombre de bactéries bénéfiques associées aux plantes. Cette recherche a montré que le tagatose est une molécule prometteuse qui pourrait être utilisée dans le développement d'un biopesticide pour le contrôle durable des maladies des cultures.

Phytophthora, sucre rare, tagatose, mode d'action, lutte Biologique

THE RARE SUGAR TAGATOSE: AN ECO-FRIENDLY FUNGICIDE AGAINST *PHYTOPHTHORA* SPECIES

Phytophthora species are among the most aggressive and widespread plant pathogens and they are causing enormous economic losses worldwide and whose control is mainly based on the use of chemicals. The overuse of chemical pesticides against these plant pathogens showed many threats to human health and the environment. An extremely scarce number of efficient eco-friendly products are available to control *Phytophthora* spp. and alternative molecules are therefore needed for sustainable agriculture. The aim of this thesis is to investigate the efficacy and the mechanism of action of the rare sugar tagatose against *Phytophthora* spp., in order to further develop eco-friendly fungicides based on this active ingredient. Tagatose inhibited mycelial growth and negatively affected mitochondrial processes in *P. infestans*, but not in *P. cinnamomi* indicating species-specific responses to this rare sugar. The growth inhibition of *P. infestans* can be ascribed to structural similarities of tagatose with fructose and to competitive inhibition of key enzymes of the sugar metabolism. On the other hand, *P. cinnamomi* was able to activate multiple adaptation pathways to modulate the cellular metabolism by the upregulation of genes involved in glucose transport, pentose metabolism, tricarboxylic acid cycle, reactive oxygen species detoxification, mitochondrial respiration and alternative respiration. In addition tagatose affected the composition of the phyllosphere bacteria and increased the number of beneficial plant-associated bacteria. This research showed that tagatose is a promising molecule that could be used in the development of an innovative biopesticide for the sustainable control of crop diseases.

Phytophthora, rare sugar, tagatose, mechanism of action, Biological control

Discipline : Sciences de la Vie et de la Santé
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Abbreviations list

OCR: oxygen consumption rate
ROS: reactive oxygen species
PAM: pea agar medium
PB: pea broth
Rpm: revolutions per minute
PR: pathogenesis-related
DAI: days after incubation
DPT: days post treatment
FLT: fluorescence lifetime
RFU: relative fluorescence units
H_2O_2: hydrogen peroxide
$Ca(OH)_2$: calcium hydroxide
H_2SO_4: sulfuric acid
CO_2: carbon dioxide
TCA: tricarboxylic acid
DEGs: differentially expressed genes
PCA: principal component analysis
GO: gene ontology
ABC: ATP-binding cassette
FLD: fluorimetric detector
BLAST: basic local alignment search tool
BiNGO: Biological Networks Gene Ontology
TAG: tagatose
F_TAG: tagatose formulation
NA: nutrient agar
CFU: colony forming unit
BCA: biological control agents
PGPB: plant growth promoting bacteria
TEM: transmission electron microscopy
SBE: sugar beet extract
GM: genetic modification
HPLC: high performance liquid chromatography
nma111: pro-apoptotic serine protease
tub-b: b-tubulin
g6pd: glucose-6-phosphate dehydrogenase
pfk: phosphofructokinase
ces: cellulose synthase
maai: maleylacetoacetate isomerase
atp4: ATP synthase subunit 4
atpB: ATP synthase subunit beta
gst: glutathione S-transferase
aif: apoptosis-inducing factor
cytc: cytochrome c
cox: cytochrome c oxidase
ndufv: NADH dehydrogenase ubiquinone flavoprotein
MOP: multidrug/oligosaccharidyl-lipid/polysaccharide

Résumé

Les espèces de *Phytophthora* comptent parmi les phytopathogènes les plus agressifs et les plus répandus dans le monde et causent d'énormes pertes économiques et dont le contrôle est principalement basé sur l'utilisation de produits chimiques. Cependant, l'utilisation excessive de pesticides chimiques contre les espèces de *Phytophthora* a fait apparaître de nombreuses menaces pour la santé humaine et l'environnement et de nombreux produits chimiques ont ainsi été bannis. L'objectif de cette thèse est d'étudier l'efficacité et le mécanisme d'action du sucre rare, le tagatose, contre les espèces de *Phytophthora* afin de permettre le développement de produits écologiques basés sur cet ingrédient actif. Le tagatose a inhibé la croissance mycélienne et a affecté négativement les processus mitochondriaux chez *P. infestans*, mais pas chez *P. cinnamomi*, indiquant des réponses différentes entre les deux espèces de *Phytophthora* vis à vis de ce sucre rare. L'inhibition de la croissance de *P. infestans* peut être attribuée aux similitudes structurelles du tagatose avec le fructose et à l'inhibition compétitive des enzymes du métabolisme des glucides. D'autre part, *P. cinnamomi* a été capable d'activer de multiples voies d'adaptation en activant les gènes impliqués dans le transport du glucose, le métabolisme des pentoses, le cycle de l'acide tricarboxylique, la détoxification des espèces réactives de l'oxygène et la respiration mitochondriale et alternative. En outre, le tagatose affecte la composition des bactéries de la phyllosphère et augmente le nombre de bactéries bénéfiques associées aux plantes. Cette recherche a montré que le tagatose est une molécule prometteuse qui pourrait être utilisée dans le développement d'un biopesticide pour le contrôle durable des maladies des cultures.

Mots clés: *Phytophthora*, sucre rare, tagatose, mode d'action, lutte Biologique

Abstract

Phytophthora species are among the most aggressive and widespread plant pathogens and they are causing enormous economic losses worldwide and whose control is mainly based on the use of chemicals. The overuse of chemical pesticides against these plant pathogens showed many threats to human health and the environment. An extremely scarce number of efficient eco-friendly products are available to control *Phytophthora* spp. and alternative molecules are therefore needed for sustainable agriculture. The aim of this thesis is to investigate the efficacy and the mechanism of action of the rare sugar tagatose against *Phytophthora* spp., in order to further develop eco-friendly fungicides based on this active ingredient. Tagatose inhibited mycelial growth and negatively affected mitochondrial processes in *P. infestans*, but not in *P. cinnamomi* indicating species-specific responses to this rare sugar. The growth inhibition of *P. infestans* can be ascribed to structural similarities of tagatose with fructose and to competitive inhibition of key enzymes of the sugar metabolism. On the other hand, *P. cinnamomi* was able to activate multiple adaptation pathways to modulate the cellular metabolism by the upregulation of genes involved in glucose transport, pentose metabolism, tricarboxylic acid cycle, reactive oxygen species detoxification, mitochondrial respiration and alternative respiration. In addition tagatose affected the composition of the phyllosphere bacteria and increased the number of beneficial plant-associated bacteria. This research showed that tagatose is a promising molecule that could be used in the development of an innovative biopesticide for the sustainable control of crop diseases.

Keywords: *Phytophthora*, rare sugar, tagatose, mechanism of action, Biological control

Le contexte et les objectifs de la thèse

Les espèces de *Phytophthora* peuvent être considérées comme le groupe d'agents phytopathogènes le plus dévastateur de la planète. Le genre *Phytophthora* comprend plus de 100 espèces avec de grandes différences dans leurs nombre d'hôtes. Par exemple *P. infestans*, n'infecte que quelques espèces de plantes alors que, *P. cinnamomi*, peut infecter jusqu'à 5000 espèces de plantes, dont beaucoup sont importantes pour l'agriculture, la foresterie et l'horticulture. Les maladies causées par *Phytophthora* sont responsables de graves pertes économiques dans la production agricole, horticole et forestière. Par exemple, le mildiou de la pomme de terre, causé par *P. infestans*, a un coût pour les producteurs estimé à environ 5 milliards de dollars par an et nécessite des applications fréquentes de produits phytosanitaires.

Au cours des dernières décennies, les mesures de lutte chimique ont été largement utilisées pour réduire les pertes de récolte causées par les agents pathogènes et les ravageurs des plantes. À l'échelle mondiale, les coûts de la gestion chimique des maladies causées par *Phytophthora* représentent plus de 25 % du marché annuel des fongicides. Les fongicides à base de phénylamide, tels que le métalaxyl, sont couramment utilisés pour lutter contre les maladies végétales causées par *Phytophthora* et sont très efficaces pour la gestion du mildiou. Cependant, l'utilisation excessive de ces produits chimiques a conduit au développement de souches résistantes. En plus, l'utilisation continue et abusive de pesticides chimiques a conduit à l'apparition de problèmes environnementaux et de santé humaine. Récemment, le nombre de produits chimiques enregistrés a diminué en raison des restrictions imposées en raison de leurs effets négatifs sur la santé humaine et l'environnement.

La génération de cultures résistantes est une alternative écologique aux méthodes chimiques et peut être la meilleure option pour gérer les maladies du *Phytophthora*. La résistance aux *Phytophthora* peut être obtenue par des méthodes de sélection variétale et des méthodes de génie génétique. Cependant, la sélection est une approche qui prend du temps, tandis que les méthodes de génie génétique sont confrontées à plusieurs problèmes législatifs.

L'utilisation d'espèces bactériennes comme alternative aux fongicides chimiques pour le contrôle des maladies des plantes a pris une importance considérable ces dernières années en raison des effets négatifs négligeables sur la santé humaine et l'environnement. De plus, des champignons tels que les espèces de *Trichoderma* ont montré des effets prometteurs pour contrôler plusieurs espèces de *Phytophthora* principalement par des mécanismes d’antagonismes. Cependant, malgré les effets prometteurs de plusieurs bactéries et

champignons contre les maladies du *Phytophthora* en laboratoire et/ou en serre, le principal problème de ces agents biologiques est leur variable efficacité en plein champ.

Parmi les nouvelles approches prometteuses en protection des plantes, les produits biologiques contenant des métabolites bactériens présentent un intérêt particulier. Par exemple, les espèces de *Streptomyces* produit des peptides de valinomycine et de coronamycine avec des effets antifongiques contre *P. capsici* et *P. cinnamomi,* respectivement. De plus, les lipopeptides produits par des bactéries ont reçu une attention considérable en raison de leurs activités spécifiques contre plusieurs pathogènes de plantes et de leurs caractéristiques structurelles. Par exemple, les lipopeptides provenant de micro-organismes tels que les *Bacillus* et les *Pseudomonas* ont montré des effets prometteurs pour le contrôle des espèces de *Phytophthora*.

Par ailleurs, des études antérieures ont montré que les glucides peuvent être utilisés dans la protection écologique des cultures. Le Chitosan et ses oligosaccharides ont reçu beaucoup d'intérêt pour une application potentielle dans l'agriculture, la biomédecine et la biotechnologie en raison de leur biocompatibilité, biodégradabilité et bioactivité. Par exemple, le Chitosan a montré des résultats prometteurs pour le contrôle de plusieurs espèces de *Phytophthora* tels que *P. capsici*, *P. infestans*, *P. parasitica*, *P. cactorum*, *P. botryose*, *P. palmivora* et *P. fragariae*.

Récemment, les sucres rares ont attiré l'attention du secteur agricole en raison de leur faible toxicité et de leur efficacité dans la lutte contre les maladies des plantes causées par un large éventail d'agents phytopathogènes. Les sucres rares ont été définis comme des monosaccharides et leurs dérivés qui existent rarement dans la nature. Les propriétés fonctionnelles et écologiques des sucres rares sont inconnues en raison de leur disponibilité limitée dans la nature. Cependant, la mise en œuvre de nouveaux procédés enzymatiques et microbiens a permis de réduire le coût de la synthèse de ces sucres et d'étendre leur utilisation dans divers domaines industriels et scientifiques, tels que l'agriculture, la nutrition humaine et la médecine.

Parmi les sucres rares, le tagatose est un cétohexose que l'on trouve à de faibles concentrations dans de nombreux aliments et qui ne présente aucun danger pour la santé humaine. Le tagatose inhibe la croissance de plusieurs phytopathogènes, tels que le mildiou de la tomate et de la pomme de terre (*Phytophthora infestans*), le mildiou de la vigne (*Plasmopara viticola*), l'oïdium de la vigne (*Erysiphe necator*), le mildiou du chou (*Hyaloperonospora parasitica*) et l'oïdium de la tomate (*Oidium violae*). Cependant, des recherches plus approfondies sont nécessaires pour clarifier ces propriétés d'inhibition de la croissance contre les

phytopathogènes. L'objectif de cette thèse est d'étudier l'efficacité et le mécanisme d'action du tagatose contre les espèces de *Phytophthora* afin de développer des fongicides innovants et écologiques basés sur cet ingrédient actif. En particulier, les réponses cellulaires, les changements métaboliques et la reprogrammation transcriptionnelle activés chez deux espèces de *Phytophthora* en réponse à un traitement par le tagatose ont été évalués. Les connaissances actuelles sur les modes d'action du tagatose contre les oomycètes phytopathogènes et son utilisation potentielle en agriculture ont été également résumées. En outre, pour mieux comprendre la persistance/stabilité du tagatose sur les feuilles des plantes, la contribution possible des microorganismes naturels de la phyllosphère dans la dégradation du tagatose a été examinée. Les résultats attendus fourniront des connaissances plus approfondies pour le développement de fongicides écologiques pour la protection durable des plantes.

TABLE OF CONTENT

INTRODUCTION

1. *Phytophthora* spp.

Phytophthora can be considered as the most devastating group of plant pathogens on earth and its name is derived from Greek "phyto" (plant) and "phthora" (destroyer) (Hardy 2004). The genus belongs to the class oomycetes in the Kingdom chromista or stramenopiles, which is phylogenetically closer to the heterokont algae than the true fungi or Mycetae (Hardy 2004). The origin of the genus *Phytophthora* is closely associated with the Irish Potato Famine (Ribeiro 2013). The famine resulted from severe potato blights in the years 1845–1846 and led to Ireland losing as many as a quarter of its eight million inhabitants to starvation and emigration (Ribeiro 2013). Until the latter part of the 20th century, *Phytophthora* species and other oomycetes were regarded by most plant pathologists to be fungi, but this idea was discarded by the power of molecular genetics that revealed an evolutionary distance between oomycetes and fungi (Fry 2008) (Figure 1).

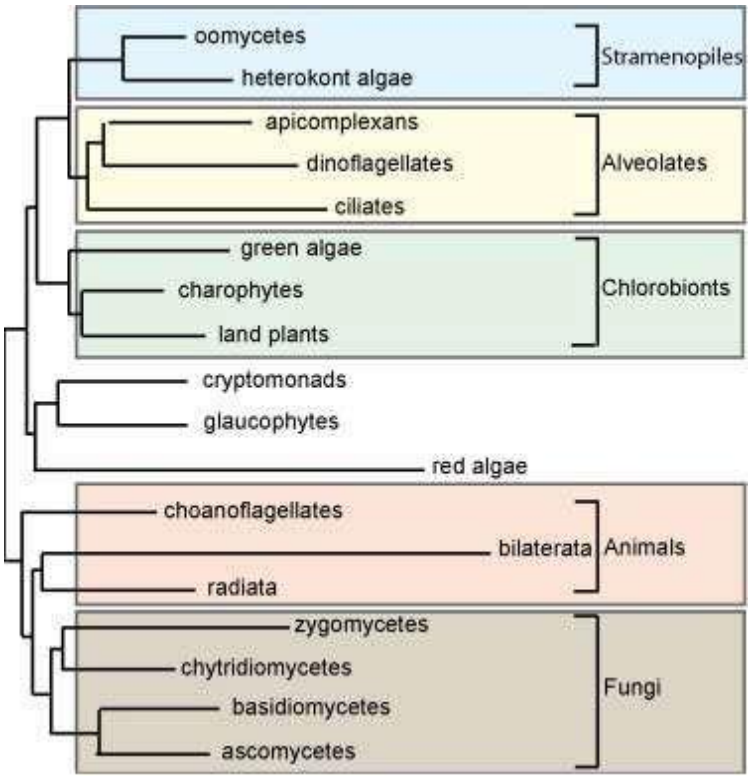

Figure 1. Phylogenetic tree showing evolutionary relationships between the major groups of eukaryotes. There isa close evolutionary relationship between the Stramenopiles (including the Oomycetes) and the Alveolates (Hardham 2005)

Phytophthora spp. are diploid, and they can reproduce asexually to produce chlamydospores or sporangia (Hardy 2004). When conditions favouring growth prevail, *Phytophthora* spp. enters the asexual sporulation cycle (Hardham 2005). Somatic hyphae form multinucleate sporangia that cleave and release 20–30 uninucleate, biflagellate zoospores (Hardham 2005). The wall-less zoospores encyst, forming walled cysts that germinate and penetrate the plant (Hardham 2005). Within 2 or 3 days in a susceptible host, sporangia will form on the plant surface and the asexual cycle may be repeated many times in quick succession, rapidly amplifying the inoculum potential in the infected area (Hardham 2005). On the other hand, the sexual reproduction occurs when a fertilization tube from the antheridium deposits its nucleus inside the oogonium leading to fusion between the two nuclei and the formation of an oospore within the oogonium (Hardy 2004) (figures 2 and 3).

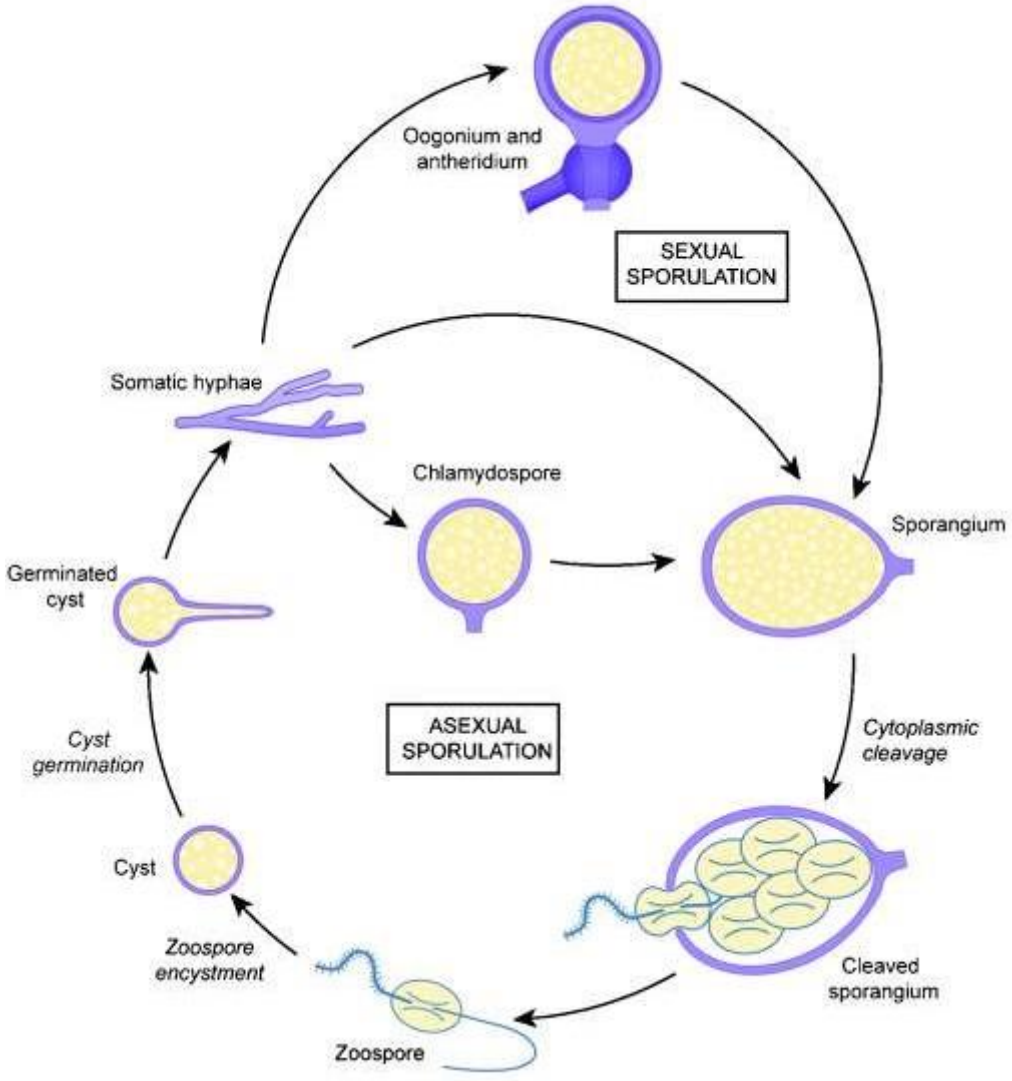

Figure 2. Life cycle of *Phytophthora* (adapted from Hardham 2005)

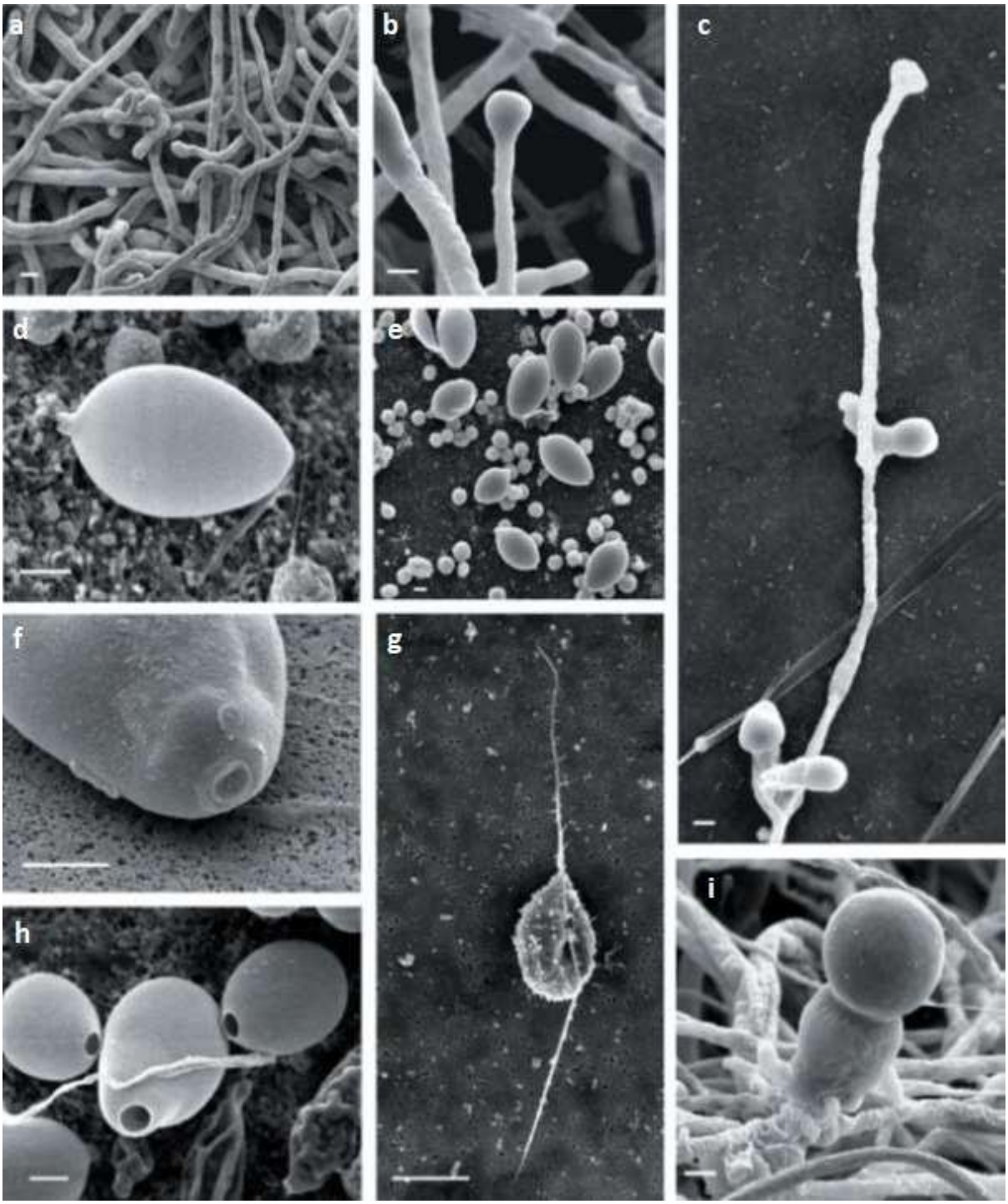

Figure 3. Stages of the spore cycles of *Phytophthora infestans*. The bar in each panel represents 5 cm. **a** | Vegetative, non-sporulating hyphae. **b** | The swollen tip of an asexual sporangiophore, typical of a sporangia initial. **c** | A sporangiophore containing four maturing asexual sporangia on lateral branches and a terminal sporangiophore initial. **d** | An ungerminated sporangium. **e** | A mixture of sporangia and zoospores. **f** | The apical tip of a sporangium, showing the opening through which zoospores are released (operculum), which is now filled with anapical plug. **g** | A zoospore with its two flagella attached to a central groove; decorations or mastigonemes can beseen on the upper flagellum. **h** | Sporangia after releasing zoospores, displaying open opercula. **i** | An oospore formed from A1 and A2 hyphae, (Judelson and Blanco 2005).

2. **Crops affected by *Phytophthora* spp. and the impact on agriculture**

The genus *Phytophthora* comprises more than 100 described species with great differences in their host ranges. For instance, some species such as *P. infestans* have a narrow host range and infects only few plant species while others such as *P. cinnamomi* have a broad host range and can infect close to 5000 species of plants, including many of importance in agriculture, forestry and horticulture (Hardham 2018). *Phytophthora* diseases caused severe economic losses in agriculture, horticultural and forest production (Fry et al., 2015; Hardham 2005; Kamoun et al.,2015). For example, potato late blight, caused by *P. infestans*, has an estimated cost for growers of about 5 billion dollars per year and requires frequent applications of plant-protection products (Judelson and Blanco 2005). Similarly, at a global scale, disease losses caused by *P. sojae* in soybean have been estimated at 1.2 billion dollars per year (Drenth and Sendall 2013) while avocado crops losses caused by *P. cinnamomi* in California has been estimated to exceed US$40 million annually (Ploetz 2013) (Table 1).

Table 1. Host ranges of important *Phytophthora* spp. (adapted from Fawke et al., 2015)

***Phytophthora* species**	**Hosts**	**Infection sites**
P. cinnamomi	Very broad range, including most annual and herbaceous perennial species	roots
P. capsici	*Capsicum annuum*, members of the Cucurbitaceae, Fabaceae, and Solanaceae	stems and fruit
P. infestans	Potato, tomato, wild tobaccos	shoots
P. palmivora	Very broad range including palm and fruit tree species, *Medicago truncatula*, *Nicotiana benthamiana*	roots, trunks, buds, leaves
P. parasitica	Very broad range, including *Solanum lycopersicum*, *Solanum tuberosum*, *Capsicum annuum*	roots and leaves
P. ramorum	Very broad range, including *Quercus agrifolia*, *Notholithocarpus densiflorus*	phloem and inner bark
P. sojae	*Glycine max*, *Glycine soja*, *Lupinus* spp	roots

Phytophthora species are considered as ‘’farmers nightmare’’ since they can transform whole fields from slightly diseased to nearly completely destroyed within just a few days (Figure 4) (Fry 2008).

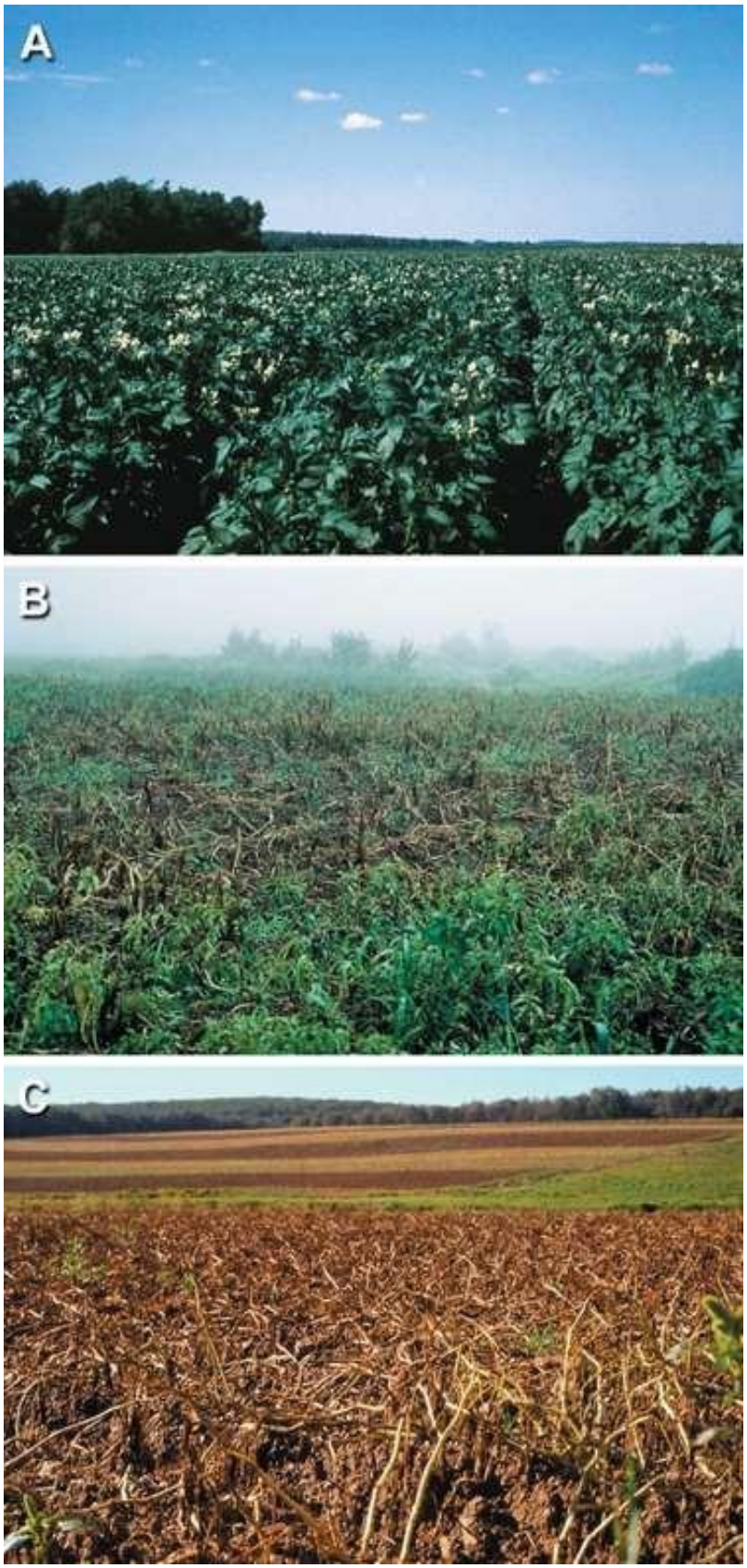

Figure 4. The aggressiveness of *Phytophthora* spp. The speed with which *P. infestans* can destroy a field of potatoes is impressive. A field that appeared 'healthy' on one week (A) can be visibly severely diseased in the nextweek (B), and within another week can be totally destroyed (C), (Fry 2008).

3. **Methods of disease control and prevention**

3.1. Chemicals

In the last decades, chemical control measures have been widely used to decrease crop losses caused by plant pathogens and pests. Fungicides have different mode of actions against plant pathogens acting by (a) impairing lipids, sterol, and other membrane components, (b) amino acids and protein synthesis, (c) signal transduction, (d) respiration, (e) mitosis and cell division, (f) nucleic acids synthesis, and (g) multisite activity (Yang et al., 2011). Worldwide, costs for chemical management of *Phytophthora* diseases represent over 25% of the annual fungicide market (Kuhajek et al., 2002). For example, over 5 million pounds of chemical active ingredient have been employed against late blight annually in the United States (Leesutthiphonchai et al., 2018), which costs farmers millions of dollars annually. In particular, several fungicides were found effective for the control of plant diseases caused by *Phytopthora* spp. (Belisle et al., 2019;Hao et al., 2019; Timodis and Tsoumoridis 2001). For example, Phosphonate (also referred to as phosphite) controls many plant diseases caused by a range of *Phytophthora* spp. *in planta* even at concentrations where the product only partially inhibit the *in vitro* growth of pathogens (Hardy 2004). However, phosphonate can cause severe phytotoxicity and plant deaths if inappropriate concentrations are applied (Hardy 2004). In addition, there is some evidence that repeated applications of phosphonate in horticultural situations lead to the development for phosphonate-tolerant isolates of *P. cinnamomi*, and these isolates also appear to be more virulent (Hardy 2004). Likewise, phenylamide fungicides, such as metalaxyl, are commonly used for the control of plant diseases caused by *Phytopthora* and are very effective for late blight management (Lalet al., 2018). However, the overuse of this chemical led to the development of resistant strains to metalaxyl (Matson et al., 2015). In addition to the emergence of resistant isolates, the continuous and abusive use of chemical pesticides led to the apparition of environmental and human health problems (Aktar et al., 2009). Pesticides have contaminated almost every part of our environment and are found in soil, air, and in surface and ground water across the countries,which represent a significant risk to non-target organisms ranging from beneficial microorganisms, to insects, fish, birds, and plants (Aktar et al., 2009). Recently, the number of registered chemical products has decreased as a result of the restrictions imposed because of their negative effects on human health and the environment (Sutton 1996).

3.2. Plant breeding and genetic engineering

Generation of resistant crops is an eco-friendly alternative to chemical methods and it can be the best option to manage *Phytophthora* diseases. The resistance to *Phytophthora* spp. could be achieved by both classical breeding and genetic modification (GM) approaches. For instance, classical breeding for resistance to *P. infestans* was focused on the *R* genes derived from *S. demissum* (Park et al., 2009). Although the introduction of R genes is a time-consuming approach, the broad-spectrum resistance conferred to wild *Solanum* species can be a promising method to combat diseases enabling more durable resistant potato cultivation (Park et al., 2009). However, the introduced *R* genes are in most cases surrounded by relatively large stretches of *S. demissum*-derived DNA (Park et al., 2009). This effect is called linkage drag, meaning that not only the *R* genes are inserted but also a great number of other alleles from the wild speciesare introduced into the new variety (Park et al., 2009). These unknown alleles often negatively influence traits of agricultural interest such as low yield or disease susceptibility (Park et al., 2009). In addition, because of the tetraploid nature and heterozygosity of potato varieties, the breeding releases a high degree of diversity, so that it is difficult to recover the exact parental phenotype in a breeding programme (Fry 2008; Jacobsen and Van der Vossen 2009). To minimize the undesirable effects of linkage drag, the Marker-Assisted Backcross Breeding approach was implemented allowing the transfer of one or more genes of interest traits from donor parent into the background of the improved variety and recover the recurrent parent genome by eliminating the undesirable genes (Hasan et al., 2015). For example, soybean breeding using the Marker-Assisted Backcross Breeding technique showed an improved host-plant resistance to *P. sojae* (Ramalingam et al., 2020). Resistant plant for some crop species subject to *Phytophthora* diseases is sometimes available, but may be limited and is subject to loss of effectiveness as the pathogen evolves methods of overcoming resistance (Hardham 2001). For example, resistant isolates of *P. infestans* have been identified overcoming all 11 *R* genes in potato (Bradshaw et al., 2006). In addition to classical breeding methods, genetic engineering methods can help plant breeders to introduce desirable genes into cultivars that are outside conventional and sexual hybridization (crossing) techniques and will complement traditional breeding efforts (Park et al., 2009). For instance resistance breeding *via* a GM approach could achieve the introduction of the R genes without the problem of linkage drag (Park et al., 2009). In the case of potato, there have been several trials to introduce GM potato varieties resistant to *P. infestans* to the world market, but they so far failed due to the changed legislation and unwillingness of large processors to process GM potatoes (Park et al., 2009). These issues are highly associated with the general sentiments of the public and other political decisions

(Park et al., 2009). Although there are still a lot of opponents in the public debate, the acreage of GM crops is dramatically increasing and the number of countries permitting the growth of GM crops is also increasing (Park et al., 2009).

3.3. Biological alternatives

3.3.1. Microorganisms

For decades, the control strategies against *Phytophthora* diseases relied solely to the application of chemical fungicides (Mizubuti et al., 2007). In addition, the continuous and abusive use of chemical fungicides has led to the development of resistance in plant pathogens and the decrease of the natural biodiversity in ecosystems (Aktar et al., 2009; Fry and Goodwin 1997; Hardy 2004). As a result, the use of living bacterial species as an alternative to chemical fungicides for the control of plant diseases is gaining a considerable importance in recent years due to the negligible negative effects on human health and the environment (Table 2). In addition to bacterial species, fungal species such as *Trichoderma* species showed promising effects for the control of several *Phytophthora* species mainly by antagonistic mechanisms (Table 3). However, despite the promising effects of several biocontrol agents found in the lab and/or greenhouse tests, the main problem of biological agents is their variable efficacy under field conditions (Axel et al., 2012).

Table 2. Bacterial biocontrol agents and their mechanism of action against *Phytophthora* spp.

Bacteria	Target pathogen	Mechanism	Reference
Bacillus megaterium KL39	*P. capsici*	Antibiosis	Jung and Kim 2005
Paenibacillus illinoisensis KJA-424		SAR/ISR	Jung et al., 2005
Penicillium striatisporum Pst10		Antibiosis	Ma et al., 2008
Serratia marcescens F-1-1		Antibiosis	Okamoto et al., 1998
Streptomyces griseus H7602		Production of lytic enzymes	Nguyen et al., 2012
Serratia plymuthica A21–4		Inhibition of mycelium growth, and zoospore formation	Shen et al., 2002
Chryseobacterium wanjuense strain KJ9C8		Colonization and antibiosis	Kim et al., 2012
Flavobacterium johnsoniae strain GSE09		Colonization of plant root	Sang and Kim 2012
Lysobacter antibioticus HS124		Production of 4-hydroxyphenylacetic acid and lytic enzymes	Ko et al., 2009
Novosphingobium capsulatum strain YJR107		Antagonism	Sang et al., 2008
Paenibacillus polymyxa GBR-462		Inhibition of mycelium growth	Kim et al., 2009
Pseudomonas fluorescens 89B61	*P. infestans*	ISR	Yan et al., 2002
Lysobacter capsici		Mycophagy	Tomada et al., 2017
Pseudomonas fluorescens 89B61		ISR	Yan et al., 2002
Cellulomonas flavigena		Antagonism	Lourenco et al., 2006
Enterobacter cloacae S11:T:07		Phenyl acetic acid, indole-3-acetic acid, tyrosol	Slininger et al., 2007
Xenorhabdus nematophilus var. pekingensis CB6		Xenocoumacin 1	Yang et al., 2011
Pseudomonas aureofaciens BS1393		Induced systemic resistance	Zakharchenko et al., 2011
Bacillus cereus		Induction of systemic resistance	Silva et al., 2004
Burkholderia cepacia strain ASPB2D	*P. nicotianae*	ISR	Coventry and Dubery 2001
Paenibacillus polymyxa GBR-462	*P. palmivora*	Inhibition of mycelium growth, and zoospore formation	Kim et al., 2009
Paenibacillus polymyxa B2, B5, B6		Antibiosis	Timmusk et al., 2009

Table 3. Fungal biocontrol agents and their mechanism of action against *Phytophthora* spp.

Fungi	Target pathogen	Mechanism	Reference
Hebeloma crustiliniforme	*P. cambivora*	Competition	Branzanti et al., 1999
Trichoderma asperellum	*P. capsici*	Antagonism	Jiang et al., 2016
Trichoderma hamatum s382		SAR	Khan et al., 2004
Gigaspora margarita		ß-1,3- glucanase, chitinase	Ozgonen et al., 2009
Hebeloma crustiliniforme,	*P. cinnamomi*	Competition	Branzanti et al., 1999
Trichoderma hamatum (F56)		Antagonism	McLeod 1995
Paxillus involutus		Competition	Branzanti et al., 1999
Laccaria laccata		Competition	Branzanti et al., 1999
Trichoderma harzianum HNA14	*P. infestans*	Mycoparasitism	Yao et al., 2015
Pseudozyma flocculosa		cis-9-Heptadecenoic Acid	Avis and Belanger 2001
Nigrospora sphaerica		Phomalactone	Kim et al., 2001
Fusarium oxysporum EF119		Antioomycete	Kim et al., 2007
Trichoderma asperellum PR11	*P. megakarya*	Competition,	Deberdt et al., 2008
Glomus mosseae	*P. nicotianae*	Competition	Trotta et al., 1996
Glomus mosseae BEG 12	*P. parasitica*	Induction of ISR	Cordier 1998
Trichoderma asperellum	*P. ramorum*	Mycoparasitism	Widmer 2014

3.3.2. Natural molecules

3.3.2.1. Proteins and Lipids

The inhibition of *Phytophthora* spp. by certain microorganisms can be done through their metabolites. For example *Streptomyces* spp. produced valinomycin and coronamycin peptides with an antifungal effects against *P. capsici* and *P. cinnamomi* respectively (Ezra et al., 2004; Lim et al., 2007). In addition to peptides from bacterial origin, peptides extracted from medicinal plants showed an inhibitory effect against *P. infestans* (Rogozhin et al., 2020). Furthermore, synthetic peptides showed an inhibitory effect against *P. infestans* and late blight symptoms development on potato leaves and tubers (Ali and Reddy 2000). Among the new approaches in plant protection, biological products containing bacterial metabolites are of particular interest (Maksimov et al., 2020). Lipopeptides are among the secondary metabolites produced by bacteria that received considerable attention due to their specific activities against various plantpathogens and their structural features (Maksimov et al., 2020). These compounds are amphiphilic, resistant to hydrolysis by peptidases and proteases, and insensitive to oxidation and the action of relatively high temperatures (Maksimov et al., 2020). For instance, Lipopeptides from microorganisms such as *Bacillus* spp. and

Pseudomonas spp. showed promising effects for the control of *Phytophthora* spp. (Tran et al., 2017; Wang et al., 2019; Wang et al., 2020). Lipopeptides have different modes of action against *Phytophthora* spp. such as the induction of plant systemic resistance (Tran et al., 2017) and the induction of *Phytophthora* cell death (Wang et al., 2019). In particular, Iturin A induces cell membrane damage, irregular organelle formation, oxidative stress, and dysfunction of mitochondria, resulting in *P. infestans* hyphal cell death (Wang et al., 2019). However, massetolide A provided a significant control of *P. infestans*, both locally and systemically *via* induced resistance (Tranet al., 2017). In addition, it has been found that the combination of two lipopeptides can have additive effects since the combination of Iturin A and Fengycin A (I + F) showed a higher efficacy in controlling potato late blight than individual lipopeptides (Wang et al., 2020).

3.3.2.2. Sugars

3.3.2.2.1. Conventional sugars

Previous studies showed that carbohydrates can be used as eco-friendly tools for crop protection. The main di- and oligosaccharides reported as elicitors of plant defences and/or resistance inducers against pathogens include sucrose, trehalose, B 1,3 glucans, fucans, carrageenans, ulvans, alginates, chitin, chitosan and oligogalacturonides (Trouvelot et al., 2014). Chitosan and its oligosaccharides have received much interest for potential application in agriculture, biomedicine and biotechnology due to their biocompatibility, biodegradability and bioactivity (Katiyar et al., 2014). Chitosan is a one of the most abundant natural amino polysaccharides extracted from the exoskeleton of crustaceans, insect and fungal cell walls (Katiyar et al., 2014) and it showed promising results for the control of various *Phytophthora* spp. such as *P. capsici, P. infestans, P. parasitica, P. cactorum, P. botryose, P. palmivora* and *P. fragariae* (Atia et al., 2005; Eikemo et al., 2003; Esyanti et al., 2019; Falcón et al., 2007; Sunpapao and Pornsuriya 2013; Sunpapao and Pornsuriya 2014). The control of *Phytophthora*spp. diseases by chitosan relies mainly on the direct inhibition of the pathogen (Esyanti et al., 2019; Sunpapao and Pornsuriya 2013; Sunpapao and Pornsuriya 2014; Zohara et al., 2019). However, other experiments showed that chitosan displays dual effects against *P. infestans* by (a) direct antifungal (fungistatic) activity such as inhibition of fungal mycelial growth, sporangia production, release of zoospores and cysts germination, and (b) induction of tomato plant defence, with an increase in peroxidase activity, generation of reactive oxygen species (ROS), accumulation of pathogenesis related (PR) proteins such as chitinase,

ß-1,3- glucanaseand PR-14 proteins, and an increase of salicylic acid contents (Atia et al., 2005). Moreover it has been shown that oligochitosan is more effective than chitosan in inhibiting mycelial growth of *P. capsici* (Xu et al., 2007). Other carbohydrates such as curdlan b-1,3-Glucooligosaccharides and carrageenans were effective in controlling diseases caused by *P. infestans* and *P. parasitica* respectively (Li et al.,2014; Mercier et al 2000). In particular the λ-carrageenan efficiently induced the signalling pathways mediated by ethylene, jasmonic acid and salicylic acid and induce defence gene expression in tobacco leaves such as sesquiterpene cyclase, chitinase and proteinase inhibitor (Mercier et al., 2000). Curdlan ß-1,3-Glucooligosaccharides exhibit an activation effect on the early- and late-defence responses in potato leaves leading to an accumulation of H_2O_2 and salicylic acid and increase of phenylalanine, amino-lyase, b-1,3-glucanase and chitinase activities (Li et al., 2014). Likewise, a sugar beet extract (SBE) from a large-scale plant waste product, induced potato resistance under green-house conditions (Laith et al., 2013). SBE had no apparent toxic effect on the germination of sporangia or on the hyphal growth of the pathogen but triggered the induction of pathogenesis-related protein (PR-1 and PR-2) indicating that carbohydrates generally act by stimulating plant resistance rather than impairing the pathogen growth (Laith et al., 2013).

3.3.2.2.2. Rare sugars

3.3.2.2.2.1. Rare sugars in general

Rare sugars are defined as monosaccharides and their derivatives with limited availability in nature (Granström et al., 2004). Among all possible hexoses and pentoses only seven exist in large amount and described as common monosaccharides (glucose, fructose, galactose, mannose, ribose, xylose and L-arabinose) whereas twenty hexoses and nine pentoses were considered as rare sugars (Izumori et al., 2008). The role of rare sugars is not yet understood and their promising biological properties are underestimated due to the limited availability of these compounds in nature (Li et al., 2013). However, the development of novel enzymatic and microbial methods lowered the production costs of rare sugars and extended their use to numerous disciplines (Izumori 2006; Li et al., 2013). In agriculture, rare sugars showed dual effects in plant disease control by inhibiting the growth of pathogens and by inducing plant resistance (Izumori et al., 2008; Kano et al., 2011). Rare sugars such as altrose, allose and psicose has been patented as growth inhibitors of filamentous fungi such *Aspergillus niger*, *Cladosporium cladosporioides* and *Penicillium chrysogenum*, as well as plant pathogenic

bacterium (Izumori et al., 2008). In addition, psicose and allose upregulated the expression of defence-related genes (e.g. lipoxygenase-2, β-1,3-glucanase, peroxidase and chitinase) in rice, leading to plant resistance induction against *Xanthomonas oryzae* (Kano et al., 2011).

3.3.2.2.2.2. Tagatose

Tagatose, is a rare natural ketohexose, an isomer of galactose and an epimer of fructose with an inversion of the spatial configuration of hydroxyl group of the fourth carbon (Espinoza and Fogelfeld 2010) (Figure 5).

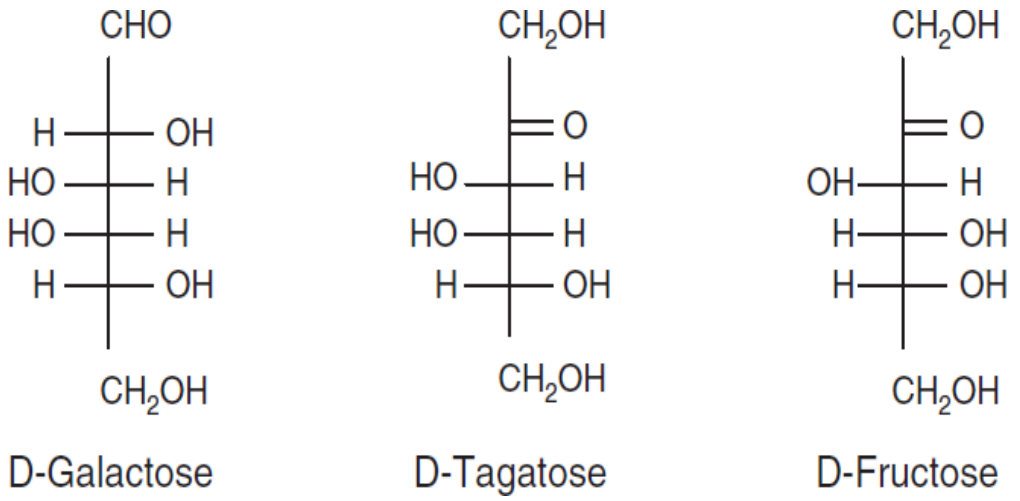

Figure 5. The biochemical structure of D-tagatose. D-tagatose is an isomer of D-galactose and an epimer of D-fructose (Espinoza and Fogelfeld 2010)

Tagatose occurs in nature at very low concentrations, and it was found within various food products, mainly dairy products and some fruits (Skytte 2006) (Table 4)

Table 4: Natural occurrence of tagatose in different food products (Skytte 2006)

Food	mg/kg	Source
Apples	3500	Eurofins, 2005[2]
Pineapples	1800	Eurofins, 2005[2]
Oranges	1500	Eurofins, 2005[2]
Cranberry concentrate	800	Eurofins, 2004[2]
Raisins	700	Eurofins, 2004[2]
Date	700	Eurofins, 2004[2]
Whole wheat	100	Eurofins, 2005[2]
Dried white beans	100	Eurofins, 2005[2]
Tropical date tree	30% of sugar	Biospherix Incorporated[3]
Sterilized cows milk	2000–3000	Troyana *et al.*[4,5]
Powdered cows milk	800	Richards and Chandrasekhara[6]
Hot cocoa with milk	140–1000	Biospherix Incorporated[3]
Ultra high temperature milk	5	Biospherix Incorporated[3]
Enfamil® infant formula	23	Biospherix Incorporated[3]
BA® nature yoghurt	29	Biospherix Incorporated[3]
Roquefort cheese	20	Biospherix Incorporated[3]
Feta cheese	17	Biospherix Incorporated[3]
Gjetost cheese	15	Biospherix Incorporated[3]
Parmesan cheese	10	Biospherix Incorporated[3]
Lactobacillus and *Streptococcus* metabolite	Variable range to be determined	Biospherix Incorporated[3]

Tagatose does not exhibit undesirable effects on human health and was ‘generally recognized as safe’ by the Food and Drug Administration and hence it is used as low-calorie sweetener due to similarity in appearance, texture sweetness and physical bulk to sucrose, (Levin 2002; Vastenavond et al., 2011) (Table 5).

Table 5: Properties of Tagatose (Jayamuthunagai et al., 2016)

Properties	Description
Common name	Tagatose
Synonyms	D-Tagatose, α-D-Tagatose, D-lyxo-hexulose
Molecular formula	$C_6H_{12}O_6$
CAS Registry	87-81-0
Classification	Carbohydrate > Monosaccharide > Keto-hexose
Structure	3 chiral Carbons; C-4 epimerised D-fructose
Mol. Wt.	180
Physical property	Anhydrous crystalline solid
Color	White
Odor	None
Taste	Intensively sweet
Solubility in water	160g/100ml at 20°C
Solubility in ethanol	0.02g/100ml at 22°C
Melting point	134°C
Heat of solution	-42.3 kJ/kg at 20°C
Decomposition Temperature	120 °C
pH stability	2-7
Sweetness	Emulates sucrose, but faster like fructose
Relative sweetness	92% of sucrose
Intestinal absorption	25% absorbed, 75% unabsorbed
Relative energy	1/3 calories of sucrose
Calorific value	0.12kcal/g
Carcinogenicity	None
Flavor enhancer	Combined flavoring with high intensity sweetening
Bulk sweetening	Yes
Humectant	Similar to sorbitol
Caramel formation	Yes, turns brown like sucrose on high temperature

In nature, the tagatose is also present as a metabolic intermediate of the tagatose-6-phosphate pathway, which is activated for the degradation of galactose and lactose in some bacteria (Bisset1974). Since tagatose is the isomeric ketose of galactose, it can be produced from any raw material sources rich in galactose by chemical and enzymatic processes (Skytte

2006). The commercial price of tagatose is expected to set close to the price of competing sugar substituting sweeteners like sorbitol (US $ 1.2/kg) and polyalcohols (US $ 3-7/kg) (Jayamuthunagai et al., 2016). Currently, tagatose is attracting attention among the researchers of different sectors because of its disease ameliorating properties (antidiabetic, obesity control, blood metabolite regulator) together with health promoting effects (anti-aging, anti-oxidant, andprebiotic). (Roy et al., 2018) (Table 6).

Table 6: Heath benefits and applications of tagatose (Oh 2007)

Health benefits	Applications
Low calorie	Low carbohydrate diets, cereals, health bars, soft drink
No glycemic effect	Diabetic food (type 2)
Anti-halitosis	Anti-hyperglycemic agent, dietary supplemen
Prebiotic	Chocolate, candy, chewing gum
Anti-biofilm, anti-plaque	Tooth paste, mouth wash
Flavor enhancement	Yogurt, bakery, milk-based drink, confectionery

In agriculture, the tagatose was patented to control important crop diseases, such as tomato and potato late blight (*P. infestans*), cucumber downy mildew (*Pseudoperonospora cubensis),* grapedowny mildew (*Plasmopara viticola*) and wheat fusarium blight (*Puccinia recondita*) (Ohara et al., 2008). Also, previous studies showed that downy mildew suppression by tagatose treatment was mainly related to a direct inhibition of *H. arabidopsidis* growth rather than *A. thaliana* resistance induction (Mochizuki et al., 2020). The tagatose showed also possible prebiotic effects on the phyllosphere microbiota and shifted the proportions of potential beneficial and potential pathogenic microorganisms by a selective nutritional and anti-nutritional effects on plant-associated microorganisms (Perazzolli et al., 2020). In addition, the tagatose showed nutritional or anti-nutritional effects within species belonging to the same genus since it supports the growth of *Trichoderma harzianum* and *T. pleuroticola* but not that of *T. pleurotum* (Komon- Zelazowska et al., 2007).

4. **Objectives**

The aim of this PhD is to investigate the efficacy and the mechanism of action of the rare sugar tagatose against *Phytophthora* spp. to further develop innovative eco-friendly fungicides based on this active ingredient. Particularly, cellular responses, metabolic changes and transcriptional reprogramming activated in two *Phytophthora* spp. in response to tagatose incubation were assessed. In addition, the current knowledge on the modes of action of tagatose against phytopathogenic oomycetes and on the potential use of this rare sugar in agriculture were summarized. Moreover, to better understand the persistence/stability of tagatose on plant leaves, the possible contribution of natural phyllosphere microorganisms in the degradation of tagatose was also examined. The expected results will provide deeper knowledge for the further development of eco-friendly fungicides for sustainable plant protection.

5. **Bibliography**

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RESULTS AND DISCUSSION

Publication 1: The rare sugar tagatose differentially inhibits the growth of *Phytophthora infestans* and *Phytophthora cinnamomi* by interfering with mitochondrial processes (published in Frontiers in Microbiology)

Résumé: Les sucres rares sont des monosaccharides et leurs dérivés qui sont rarement présents dans la nature. Les propriétés biologiques des sucres rares ne sont pas entièrement élucidées, en raison de leur disponibilité limitée dans la nature. Cependant, la mise en œuvre de procédés industriels enzymatiques et microbiens a réduit le coût de la synthèse des sucres rares et a rendu plus accessibles les études scientifiques et les applications technologiques de ces sucres. Parmi ces sucres rares, le tagatose naturellement trouvé à de faibles concentrations dans de nombreux aliments, inhibe la croissance de certains agents phytopathogènes et a été suggéré comme une alternative possible aux fongicides chimiques. Cependant, le mécanisme d'action de ce sucre rare est encore inconnu et des recherches supplémentaires sont nécessaires pour clarifier ses propriétés d'inhibition de la croissance sur les agents phytopathogènes. Les espèces de *Phytophthora* causant de graves pertes économiques en agriculture ont été choisies pour clarifier le mode d'action du tagatose. La croissance de *Phytophthora infestans* a été inhibée par 5 g/L de tagatose après 4 et 10 jours d'incubation *in vitro* tandis que la croissance de *P. cinnamomi* n'a pas été affectée par la même concentration. De même, la structure des mitochondries a été sévèrement altérée dans les échantillons de *P. infestans* incubés avec du tagatose, montrant une réorganisation des crêtes mitochondriales après 4 jours d'incubation conduisant à l'apparition de crêtes circulaires et concentriques après 10 jours d'incubation. A l'inverse, chez *P. cinnamomi*, le tagatose n'a pas affecté les structures cellulaires après 4 jours d'incubation par rapport au contrôle et seules de légères altérations mitochondriales ont été observées après 10 jours d'incubation. De même, le tagatose affecte négativement les activités mitochondriales chez *P. infestans* mais pas chez *P. cinnamomi*. En particulier, le tagatose a diminué la teneur en ATP et la consommation d'oxygène chez *P. infestans*, mais pas chez *P. cinnamomi*, par rapport aux témoins respectifs. En conséquence, la génération des espèces réactives de l'oxygène a été augmentée par le tagatose chez *P. infestans* mais pas chez *P. cinnamomi*. De plus, le tagatose a modulé différemment l'expression des gènes de *P. infestans* et de *P. cinnamomi*. Plus précisément, les niveaux d'expression des gènes liés à l'apoptose (*aif* et *nma111*) et à la réponse au stress oxydatif (*maai*) ont été augmentés par le tagatose chez *P. infestans*. En revanche, aucun de ces gènes n'a été impacté chez *P. cinnamomi*, à l'exception de la répression de l'expression de *nma111*. Ces résultats montrent que le tagatose peut être utilisé pour le développement de produits phytosanitaires pour lutter contre *P. infestans*.

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ORIGINAL RESEARCH
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The Rare Sugar Tagatose Differentially Inhibits the Growth of *Phytophthora infestans* and *Phytophthora cinnamomi* by Interfering With Mitochondrial Processes

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Rare sugars are monosaccharides with limited availability in nature and their biological functions are largely unknown. Among them, tagatose was developed as a low-calorie sweetener and showed beneficial effects on human health. Tagatose is metabolized by only certain microbial taxa and inhibits the growth of important crop pathogens (e.g., *Phytophthora infestans*), but its mode of action and the microbial responses are unknown. The aim of this study was to understand the tagatose mode of action against *Phytophthora* spp., with the final aim of developing new plant protection products. Tagatose inhibited *P. infestans* growth *in vitro* and caused severe ultrastructural alterations, with the formation of circular and concentric mitochondrial cristae. Decreased ATP content and reduced oxygen consumption rate (OCR) were found in tagatose-incubated *P. infestans* as compared to the control, with the consequent accumulation of reactive oxygen species (ROS) and induction of genes related to apoptosis and oxidative stress response. On the other hand, tagatose did not, or only slightly, affect the growth, cellular ultrastructure and mitochondrial processes in *Phytophthora cinnamomi*, indicating a species-specific response to this rare sugar. The mode of action of tagatose against *P. infestans* was mainly based on the inhibition of mitochondrial processes and this rare sugar seems to be a promising active substance for the further development of eco-friendly fungicides, thanks to its anti-nutritional properties on some phytopathogens and low risk for human health.

Keywords: rare sugar, *Phytophthora* spp., biological control, mitochondrial alteration, antioomycete activity, oxidative stress

INTRODUCTION

Rare sugars are monosaccharides and their derivatives that rarely exist in nature (Granström et al., 2004). The ecological role of rare sugars is not fully understood and their promising biological properties are underestimated, mainly due to their limited availability in terms of quantity in nature (Li et al., 2013). The implementation of industrial enzymatic and microbial processes lowered the cost of rare sugar synthesis (Granström et al., 2004; Izumori, 2006; Oh, 2007) and made scientific studies and technological applications of these carbohydrates more accessible (Oh, 2007; Li et al., 2013). Twenty hexoses (e.g., tagatose, allose, gulose, and sorbose) and nine pentoses (e.g., lyxose, xylulose, and xylitol) have been classified as rare sugars by the international society of rare sugars (Ahmed, 2001; Jayamuthunagai et al., 2017). Among them, tagatose is a ketohexose that was found naturally at low concentration (<3 mg/g) in many foods, such as apples, oranges, and milk (Vastenavond et al., 2011). Tagatose was "generally recognized as safe" by the Food and Drug Administration as it does not have negative impacts on human health (Levin, 2002; Vastenavond et al., 2011). Thanks to its safety for human health, reduced caloric value and physical properties similar to those of sucrose (sweetness, color, and texture), tagatose was approved for use as low-calorie sweetener in several countries, European Union and United States included (Vastenavond et al., 2011).

Tagatose also shows beneficial effects and therapeutic properties on humans and it was proposed for the treatment of "type 2" diabetes, hyperglycemia, anemia, and hemophilia (Levin, 2002). Moreover, tagatose affects the growth of human-associated microorganisms, inhibiting biofilm formation and co-aggregation of the oral bacteria (streptococci and actinomycetes) responsible for dental plaque formation (Levin and Lu, 2007). In particular, prebiotic properties on the human gut microbiome were attributed to tagatose, for exampleit increases the abundance of beneficial bacteria, such as *Enterococcus* spp. and *Lactobacillus* spp. (Bertelsen et al., 1999; Vastenavond et al., 2011; Hasibul et al., 2018). On the other hand, tagatose inhibits the growth of human pathogenic bacteria, such as *Streptococcus mutants* and *Salmonella enterica* serovar Typhimurium (Lobete et al., 2017; Hasibul et al., 2018). Likewise, tagatose is not catabolized by some human pathogens, such as *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Yersinia enterocolitica* (Bautista et al., 2000), indicating its nutritional or anti-nutritional effects on specific microbial taxa. Tagatose can be utilized as a carbohydrate source by only certain microbial taxa, such as *Exiguobacterium* spp., *Lactobacillus* spp., and *Lactococcus* spp. (Raichand et al., 2012; Martinussen et al.,2013; Van Der Heiden et al., 2013; Wu and Shah, 2017).In particular, tagatose can be transported into microbial cells by the phosphotransferase uptake systems and used as an intermediate in the lactose, galactose, and galactitolcatabolism by some bacterial species (Van Der Heiden et al., 2013). For example, the *Lactobacillus* spp. and *Lactococcus* spp. metabolism includes the tagatose-6-phosphate pathway (Martinussen et al., 2013; Wu and Shah, 2017) and the incubation of *Lactobacillus rhamnosus* with tagatose triggered a complex transcriptional reprograming of the carbohydrate metabolism with activation of the phosphotransferase system (Koh et al., 2013).

In plants, tagatose inhibits the growth of some phytopathogens and it was patented to control importantcrop diseases, such as tomato and potato late blight (*P. infestans*), cucumber downy mildew (*Pseudoperonospora cubensis*), grape downy mildew (*Plasmopara viticola*), cucumber powdery mildew (*Sphaerotheca fuliginea*), wheat fusarium blight (*Puccinia recondita*), cabbage downy mildew (*Peronospora parasitica*), rice and cucumber damping-off disease (*Pythium graminicola* and *Pythium aphanidermatum*) (Ohara et al., 2008). Among them, *P. infestans* causes severe economic losses on potato, tomato, and eggplant (Fry et al., 2015) and the *Phytophthora* genus comprises some of the most aggressive and widespread plant pathogens (Kamoun, 2000). For example, *Phytophthora cinnamomi* causes considerable damage to agricultural, horticultural and forest plants, with more than 3000 host species, including avocado, chestnut, and pineapple (Hardham, 2005).

Potato late blight, caused by *P. infestans*, has an estimated cost for growers of about 5 billion dollars per year and requires frequent applications of plant-protection products (Judelson and Blanco, 2005) with a consequent negative impact on human health and the environment (Fantke et al., 2012). Thus, tagatose has been previously suggested as a possible alternative to synthetic chemical fungicides (Oharaet al., 2008), thanks to the absence of deleterious effectson human health (Levin, 2002; Vastenavond et al., 2011). Tagatose showed also possible plant prebiotic effects on the phyllosphere microbiota and modified the balance of potential pathogenic and potential beneficial microorganisms by selective nutritional and anti-nutritional properties for some specific microbial taxa (Perazzolli et al., 2020). However, deeper investigations are required to clarify the growth inhibition properties of tagatose on phytopathogens, because the mechanism of action is still unknown. In addition, tagatose did not inhibit the mycelial growth of *Aspergillus niger*, *Cladosporium cladosporioides*, and *Penicillium chrysogenum* (Izumori et al., 2008) and it promoted the spore germination of *A. niger* (Hayer et al., 2013), indicating the absence of growth inhibition on some plant-associated microorganisms. More specifically, tagatose supported the growth of *Trichoderma harzianum* and *Trichoderma pleuroticola*, but not that of *Trichoderma pleurotum* (Komon-Zelazowska et al., 2007), indicating nutritional or anti-nutritional effects also within species belonging to the same genus. The variability in the response of plant-associated microorganisms to tagatose requires more information on its physiological and molecular effects, to further develop innovative biopesticides based on this active substance. The aim of this study was to clarify the mode of action of tagatose and the cellular responses in two phytopathogenic *Phytophthora* spp. *in vitro*, in order to provide deeper knowledge for the further development of eco-friendly fungicides for sustainable plant protection.

MATERIALS AND METHODS

Biological Material, Growth Conditions and Treatments

Phytophthora infestans strain VB3 and *P. cinnamomi* strain CBS 144.22 were stored in glycerol at −80°C in the fungal collection of the Fondazione Edmund Mach, Italy, and they are freely available upon request. *P. infestans* and *P. cinnamomi* were grown in Petri dishes on pea agar medium (PAM, 12.5% frozen peas and 1.2% agar in distilled water) at 18 ± 1 and 25 ± 1°C, respectively (Puopolo et al., 2014).

The *P. infestans* and *P. cinnamomi* mycelial suspension was prepared by collecting small mycelial fragments from 4-days-old colonies. Briefly, Petri dishes of *P. infestans* or *P. cinnamomi* colonies were filled with 2 mL pea broth (PB, 12.5% frozen peas in distilled water), small mycelia fragments were scraped with a sterile spatula and the mycelial suspension was filtered using a sterile Pasteur pipette containing a fine mesh. The liquid culture of *P. infestans* or *P. cinnamomi* was obtained in 10 mL PB inoculated with 100 µL of the mycelial suspension and incubated at 18 ± 1 and 25 ± 1°C under orbital shaking at 80 rpm, respectively.

The stock solution (50 g/L in distilled water) of each rare sugar, such as tagatose (Bi-PA, Londerzeel, Belgium), psicose (Carbosynth, Compton, United Kingdom), and sorbose (Carbosynth), was filter sterilized and added at the appropriate final concentration (5 or 10 g/L) in PAM or PB shortly before *Phytophthora* spp. inoculation. Filter sterilized oligomycin (Sigma-Aldrich, St. Louis, MO, United States) was used as control treatment at the final concentration of 10 µg/mL, since it is known as a growth inhibitor of fungi, such as *Aspergillus* spp., *Candida* spp., and *Penicillium* spp. (Eliskases-Lechner and Prillinger, 1996), through inhibition of ATP synthase activity (Manfredi et al., 2002; Kabala et al., 2014) and mitochondrial respiration (Galloway et al., 2012; Kooragayala et al., 2015).

Assessment of Rare Sugar Impact on *Phytophthora* spp. Radial Growth

Phytophthora spp. plugs (5 mm diameter) were cut from the edge of 14-days-old colonies and a plug was placed at the center of each Petri dish (90 mm diameter) on PAM augmented with the appropriate concentration of the rare sugar. The radial growth of *P. infestans* and *P. cinnamomi* was assessed 4 and 10 days after incubation (DAI) at 18 ± 1 and 25 ± 1°C, respectively, calculated as the average of the two perpendicular diameters of the colony, minus the plug diameter and the result divided by two. Plugs of *P. infestans* and *P. cinnamomi* mycelia developed on PAM in the presence of tagatose were transferred on new PAM dishes and the growth was then monitored as reported above. Ten replicates (dishes) were used for each treatment and the experiment was carried out twice.

Ultrastructural Analysis by Transmission Electron Microscopy

The *P. infestans* and *P. cinnamomi* liquid cultures were collected at 4 and 10 DAI in PB in the absence (control) and presence of 5 g/L tagatose or 10 µg/mL oligomycin, for transmission electron microscopy (TEM) analysis. Each mycelial sample (0.3 cm^3) was transferred into a 2 mL tube and incubated with 500 µL of fixing solution (3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) (Zuppini et al., 2010) for 1 h at room temperature under rotary shaking at 15 rpm and then for 15 h at 4°C. Samples were washed three times in 500 µL of cacodylate buffer (0.1 M, pH 7.4), post-fixed for 2 h with 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) and dehydrated in graded ethanol series of 25, 50, 75, and 100% (v/v) with an incubation of 15 min at room temperature for each ethanol concentration (Zuppini et al., 2010). Samples were infiltrated in 1:3 (v/v) araldite resin:propylene oxide (Sigma-Aldrich) by incubating for 1 h at 40°C, followed by 1:1 (v/v) araldite resin:propylene oxide for 1 h at 40°C and 3:1 araldite resin:propylene oxide overnight at 40°C. Samples were subsequently embedded in 100% araldite resin by incubating for 24 h at 40°C and for 72 h at 60°C. Ultra-thin sections (70 nm) were obtained on a Reichert-Jung ultramicrotome (Leica Biosystems, Wetzlar, Germany) and mounted on uncoated copper grids. Sections were then stained with 1% uranyl acetate (in 50% ethanol) for 15 min and 1% lead citrate for 7 min. Observations were carried out with a Tecnai G^2 transmission electron microscope (Field Electron and Ion Company, Hillsboro, OR, United States) operating at 100 kV and equipped with a Osis Veleta camera (Olympus, Tokyo, Japan). Two replicates (tubes) were analyzed for each treatment and time point and the experiment was carried out twice.

Assessment of the ATP Content

The *P. infestans* and *P. cinnamomi* mycelial suspension (200 µL) was incubated for 24 h in PB in the absence (control) and presence of 5 g/L tagatose or 10 µg/mL oligomycin in white 96-well microplate with clear flat bottom (Corning, New York, NY, United States) under orbital shaking at 80 rpm at 18 ± 1 and 25 ± 1°C, respectively. Cellular ATP content was quantified using an ATPlite luminescence assay kit (Perkin Elmer, Waltham, MA, United States) according to manufacturer's instructions (Lis et al., 2016). Briefly, each mycelial suspension was supplemented with 100 µL of lysis buffer under vigorous shaking at 700 rpm for 10 min and 100 µL were then transferred into a 96-well microplate with a transparent flat bottom (Costar, Corning) for the subsequent protein quantification. In each well, 50 µL of substrate solution (luciferin and luciferase) were added after discarding 50 µL of each sample. The luminescence resulting from the reaction of ATP was measured using a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, United States). An ATP standard curve (0.001, 0.01, 0.1, 1, 10, 100, and 1000 nmol/L) was used as reference to calculate the ATP content.

For protein quantification, each sample (100 µL) was mixed with 100 µL Bradford reagent (Pierce Coomassie Plus, Thermo Fisher Scientific), the 96-well microplate was incubated for 10 min at room temperature and the absorbance at 595 nm was measured with a Synergy 2 Multi-Mode Microplate Reader (Biotek). A standard curve of bovine serum albumin (Sigma-Aldrich; 0, 0.01, 0.05, 0.1, and 0.2 mg/mL) was used as reference to determine the protein concentration of each sample and the ATP content was then expressed per unit of total

proteins (nmol/mg) (Smith et al., 2016). Three replicates (wells) were assessed for each treatment and the experiment was carried out twice.

Assessment of the Oxygen Consumption Rate

The *P. infestans* and *P. cinnamomi* mycelial suspension (100 µL) was incubated for 16 h in PB in the absence (control) and presence of 5 g/L tagatose or 10 µg/mL oligomycin in a black 96-well microplate with Corning under orbital shaking at 80 rpm at 18 ± 1 and 25 ± 1°C, respectively. The oxygen consumption rate (OCR) was measured using the MitoXpress Xtra Oxygen Consumption Assay (Luxcel Biosciences, Agilent, Santa Clara, CA, United States) fluorescent probe for the real-time analysis of cellular respiration (Calmes et al., 2015). An aliquot (50 µL) of the liquid media was removed from each well by aspiration with a micropipette and 50 µL of fresh PB containing 100 nM MitoXPress were added and overlaid with 100 µL of mineral oil, to exclude ambient air. Oxygen depletion in the medium was assessed as the increase in the fluorescence lifetime (FLT) of the probe, using a Synergy 2 Multi-Mode Microplate Reader equipped with a time-resolved fluorescence head (Biotek). The relative fluorescence units (RFU) (340 nm excitation, 605–705 nm emission) were recorded twice for 30 µsec, with a delay of 40 µsec (after 30 and 70 µs) at 0, 4, and 8 h of reaction time at 18 ± 1 and 25 ± 1°C for *P. infestans* and *P. cinnamomi* under orbital shaking at 80 rpm, respectively. The FLT of each sample was calculated based on the RFU with 40 µsec delay (Calmes et al., 2015) as follows:

$$\text{FLT}\ (\mu\text{sec}) = (40)/\text{Ln}\ (\text{RFU1}/\text{RFU2})$$

where, 40 µsec is the delay time between the two measurements; RFU1 is the signal measured after 30 µsec and RFU2 is the signal measured after 70 µsec. Three replicates (wells) were assessed for each treatment and the experiment was carried out twice.

Quantification of Intracellular Reactive Oxygen Species

The *P. infestans* and *P. cinnamomi* mycelial suspension (100 µL) was incubated for 16 h in PB in the absence (control) and presence of 5 g/L tagatose in a black 96-well microplate with Corning under orbital shaking at 80 rpm at 18 ± 1 and 25 ± 1°C, respectively. As control treatment, 2 mM H_2O_2 was added to increase reactive oxygen species (ROS) generation, as previously reported for *Aspergillus fumigatus* (Shekhovaet al., 2017). Intracellular ROS were quantified with $2^0,7^0$-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Thermo Fisher Scientific) as previously described (Shekhova et al., 2017) with slight modifications. Briefly, 1 µL H_2DCF-DA (300 µM) was added to each well and the 96-well microplate was incubated for 1 h in the dark under orbital shaking at 80 rpm, at 18 ± 1 and 25 ± 1°C for *P. infestans* and *P. cinnamomi*, respectively. The mycelial suspension was centrifuged at 200 rpm for 2 min, 50 µL of the liquid media were removed by aspiration with a micropipette and replaced with 50 µL of fresh PB to remove the excess of unreacted fluorescent probe. Intracellular ROS were quantified at 0, 1, and 2 h of reaction time by measuring the fluorescence intensity using a Synergy 2 Multi-Mode Microplate Reader (Biotek) with an excitation filter at 485 nm and an emission filter at 530 nm, at 18 ± 1°C for *P. infestans* and at 25 ± 1°C for *P. cinnamomi* (Wong et al., 2018). Three replicates (wells) were assessed for each treatment and the experiment was carried out twice.

Primer Design for Gene Expression Analysis

The *Phytophthora* spp. gene markers related to sugar metabolism, respiration process, oxidative stress response and apoptosis were selected for quantitative real-time PCR (qPCR) analysis. For each gene, a primer pair compatible for the *P. infestans* and *P. cinnamomi* sequence was designed on conserved coding regions (**Supplementary Table S1**) and PCR products were sequenced on both strands using an AB3730xl instrument (Applied Biosystems, Thermo Fisher Scientific) at the sequencing platform facility of Fondazione Edmund Mach as validation.

RNA Extraction and Gene Expression Analysis

The *P. infestans* and *P. cinnamomi* mycelium was collected at 4 and 10 DAI in PAM covered with sterile cellophane layers in the absence (control) and presence of 5 g/L tagatose at 18 ± 1 and 25 ± 1°C, respectively. Samples were immediately frozen in liquid nitrogen, stored at −80°C and crushed using a mixer mill disruptor (MM200, Retsch, Haan, Germany) at 25 Hz for 45 s with sterile steel jars and beads refrigerated in liquid-N_2. Total RNA was extracted from 100 mg of ground *Phytophthora* spp. mycelium using the Spectrum Plant total RNA kit (Sigma-Aldrich). RNA was quantified by NanoDrop 8000 (Thermo Fisher Scientific, Wilmington, DE, United States), treated with DNase I (Invitrogen, Thermo Fisher Scientific) and the first strand cDNA was synthesized from 1 µg of total RNA using Superscript III (Invitrogen, Thermo Fisher Scientific) and oligo-dT primer. qPCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific) and specific primers (**Supplementary Table S1**) using the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) as previously described (Perazzolli et al., 2011). Briefly, the PCR conditions were: 50°C for 2 min and 95°C for 2 min as initial steps, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analyzed to verify the specificity of each amplification reaction. Three housekeeping genes were analyzed, β-tubulin (*tub-b*) (Yan and Liou, 2006), exosome complex exonuclease subunit *Rrp42* and exosome complex exonuclease subunit *Rrp43* (also called exosome ribonuclease) (Judelson et al., 2008), and their stability was validated using the ΔCt method described by Silver et al. (2006). Briefly, a qPCR was carried out for the three housekeeping genes on all samples and *tub-b* was selected as constitutive gene for normalization, because *tub-b* expression was not affected by the treatments (i.e., lowest standard deviation among the housekeeping genes tested). For the gene expression analysis, Light Cycler 480 SV1.5.0

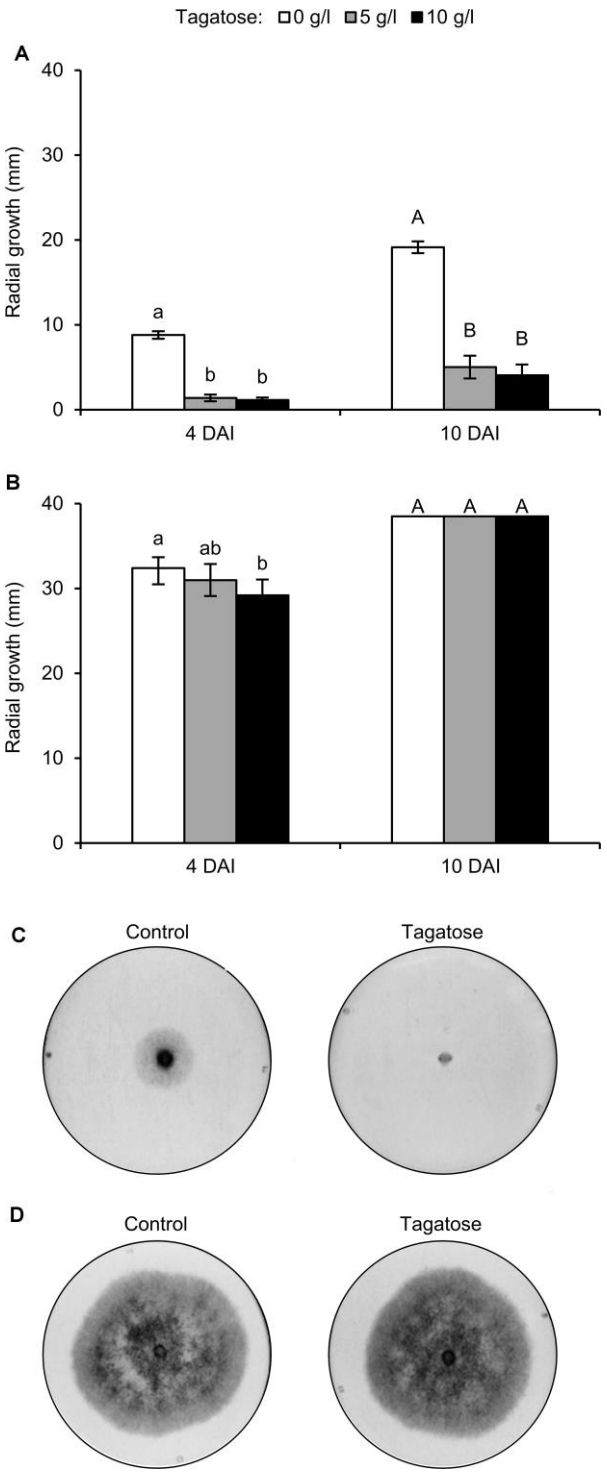

FIGURE 1 | Effect of tagatose on *Phytophthora* spp. growth. *Phytophthora infestans* (A) and *P. cinnamomi* (B) growth (mm) was assessed 4 and 10 days after incubation (DAI) on pea agar medium in the absence (white, Control) and presence of 5 (gray), or 10 g/L (black) tagatose. The radial growth was calculated as the average of the two perpendicular diameters of the colony, minus the plug diameter and the result divided by two. The Kruskal–Wallis test showed no significant differences between the two experimental repetitions *(Continued)*

FIGURE 1 | Continued
($P > 0.05$, 10 replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of 20 replicates (dishes) from the two experiments are presented for each treatment. Different lowercase and uppercase letters indicate significant differences among treatments at 4 and 10 days after incubation (DAI) according to the Kruskal–Wallis test ($P \leq 0.05$), respectively. Representative pictures of *P. infestans* (C) and *P. cinnamomi* (D) growth 4 DAI in the absence (Control) and presence of 5 g/L tagatose are shown.

software (Roche) was used to extract Ct values based on the second derivative calculation and the LinReg software version 11.0 was used to calculate reaction efficiencies for each primer pair (Ruijter et al., 2009). The relative expression level (fold change) of each gene was then calculated according to the Pfaffl equation (Pfaffl, 2001) for tagatose-incubated samples as compared to the respective control samples (calibrator) for each time point and *Phytophthora* spp., using *tub-b* as constitutive gene for normalization. Five replicates (dishes with 10 plugs in each dish) were assessed for each treatment and the experiment was carried out twice.

Statistical Analysis

All experiments were carried out twice and data were analyzed with Statistica 13.1 software (Dell, Round Rock, TX, United States). Normal distribution (Kolmogorov–Smirnov test, $P > 0.05$) and variance homogeneity of the data (Levene's tests, $P > 0.05$) were checked and parametric tests were used when both assumptions were respected. Each experimental repetition was analyzed singularly and a two-way analysis of variance (ANOVA) was used to demonstrate non-significant differences between the two experiments ($P > 0.05$). Data from the two experimental repetitions were pooled and significant differences were assessed with the Student's *t*-test ($P \leq 0.05$) or Tukey' test ($P \leq 0.05$) in case of pairwise or multiple comparisons, respectively. Fold change values of gene expression analysis were transformed using the equation $y = \mathrm{Log}_{10}(1+x)$ (Casagrande et al., 2011). When parametric assumptions were not respected, the Kruskal–Wallis test was used to demonstrate non-significant differences between the two experimental repetitions ($P > 0.05$), then data from the two experiments were pooled and a Kruskal–Wallis test was used to detect significant differences among treatments ($P \leq 0.05$).

RESULTS

Tagatose Differentially Inhibits *Phytophthora infestans* and *P. cinnamomi* Growth and Causes Ultrastructural Alterations

Phytophthora infestans growth was inhibited by tagatose at 4 and 10 DAI and the level of inhibition using 5 and 10 g/L tagatose was comparable at each time point (**Figure 1A**). Conversely, *P. cinnamomi* growth was not affected by 5 and 10 g/L tagatose at 10 DAI, and only a slight inhibition was observed with 10 g/L tagatose at 4 DAI (**Figure 1B**). When transferred to new PAM

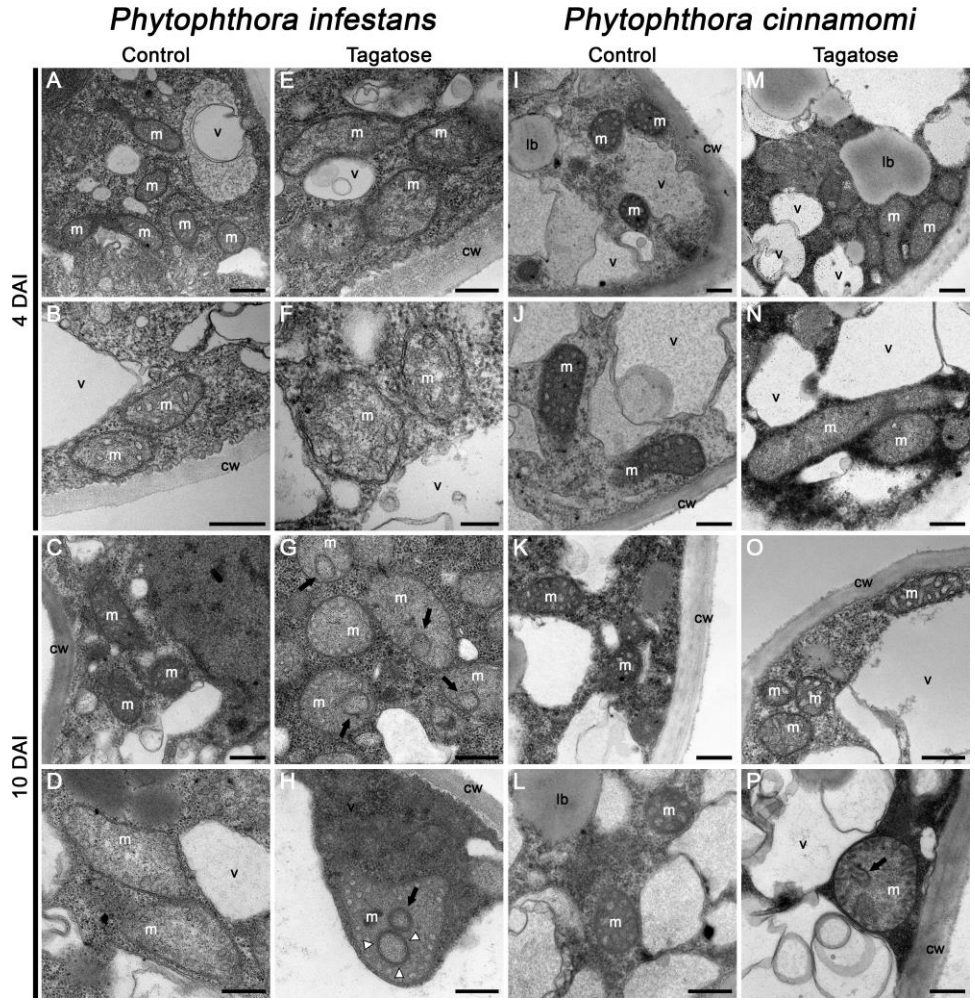

FIGURE 2 | Effect of tagatose on *Phytophthora* spp. ultrastructure. Transmission electron microscopy observations of *P. infestans* (A–H) and *P. cinnamomi* (I–P) liquid culture were carried out 4 and 10 days after incubation (DAI) in pea broth either in the absence (Control) or the presence of 5 g/L tagatose. Two replicates (tubes) were analyzed for each treatment and the experiment was carried out twice with similar results. Circular (black arrows) and concentric (white arrowheads) mitochondrial cristae are indicated. cw, cell wall; lb, lipid bodies; m, mitochondria; v, vacuoles. Bars correspond to 500 nm (A–E, G–P) or 200 nm (F). Two representative pictures are reported for each treatment and time point as follow: A,B: *P. infestans* control 4 DAI; C,D: *P. infestans* control 10 DAI; E,F: *P. infestans* tagatose 4 DAI; G,H: *P. infestans* tagatose 10 DAI; I,J: *P. cinnamomi* control 4 DAI; K,L: *P. cinnamomi* control 10 DAI; M,N: *P. cinnamomi* tagatose 4 DAI; O,P: *P. cinnamomi* tagatose 10 DAI.

dishes, the growth of *P. infestans* plugs collected at 10 DAI with 5 and 10 g/L tagatose was comparable to the growth of *P. infestans* plugs collected at 10 DAI from control dishes (data not shown), indicating that the effect of tagatose was reversible.

In order to verify the specificity of tagatose, isomers were tested, such as fructose, psicose, and sorbose. The incubation with 5 g/L fructose, 5 g/L psicose, or 5 g/L sorbose did not inhibit *P. infestans* and *P. cinnamomi* growth at 4 DAI (**Supplementary Figure S1**) and 10 DAI (data not shown). Because the minimum dosage showing a differential effect on *Phytophthora* spp. growth was 5 g/L tagatose (**Figures 1C,D**), this quantity was selected for the following experiments.

In order to investigate morphological impacts of tagatose incubation on cellular structures, TEM analyses were carried out. The typical ultrastructure of *Phytophthora* spp. (Xu et al., 2007) was observed by TEM analysis of *P. infestans* collected at 4 DAI (**Figures 2A,B**) and 10 DAI in PB (**Figures 2C,D**). Conversely, the structure of mitochondria was severely altered in tagatose-incubated *P. infestans* samples, displaying the reorganization of mitochondrial cristae at 4 DAI (**Figures 2E,F**) that leads to circular and concentric cristae at 10 DAI (**Figures 2G,H**). In the case of *P. cinnamomi*, tagatose did not affect cellular structures at 4 DAI (**Figures 2M,N**) as compared to the control (**Figures 2I–L**). Slight mitochondrial alterations, consisting of a less dense mitochondrial matrix with a sporadic occurrence of circular cristae, were found in *P. cinnamomi* only at 10 DAI (**Figures 2O,P**). The ATP synthase inhibitor oligomycin (Manfredi et al., 2002; Kabala et al., 2014), known to impair fungal growth (Eliskases-Lechner and Prillinger, 1996) and mitochondrial respiration (Galloway et al., 2012; Kooragayala

et al., 2015), caused severe mitochondrial alterations in both *P. infestans* and *P. cinnamomi* already at 4 DAI and showed either the disappearance or profound rearrangement of the mitochondrial cristae (**Supplementary Figure S2**). Taken together, these observations indicated that tagatose altered the mitochondrial structure of *P. infestans* with consequent inhibition of radial growth.

Tagatose Negatively Affects Mitochondrial Activities in *Phytophthora infestans* and Not *P. cinnamomi*

Since mitochondrial cristae alterations have been associated with the dysfunction of ATP synthase activity in yeast (Paumard et al., 2002; Gavin et al., 2004; Weimann et al., 2008), the ATP content of *Phytophthora* spp. was assessed. Tagatose decreased the ATP content of *P. infestans* as compared to the control and the effect was comparable to that obtained with the ATP synthase inhibitor oligomycin (**Figure 3A**). The ATP content of *P. cinnamomi* was not affected by tagatose and it was decreased by only the ATP synthase inhibitor oligomycin (**Figure 3B**).

The ATP synthase inhibition has been commonly linked to dysfunctions of the OCR (Galloway et al., 2012; Kooragayala et al., 2015) and ROS homeostasis (Martinez-Reyes and Cuezva, 2014) of eukaryotic cells. In *P. infestans*, we found that the OCR was inhibited by tagatose at 4 and 8 h reaction time and the FLT of tagatose-incubated samples was intermediate between that of control samples and samples incubated with the ATP synthase inhibitor oligomycin (**Figure 4A**). Conversely, *P. cinnamomi* OCR was not affected by tagatose and it was impaired by only the ATP synthase inhibitor oligomycin (**Figure 4B**). As possible consequence of ATP synthase and OCR inhibition, the ROS generation was increased by tagatose in *P. infestans* (**Figure 4C**), but not in *P. cinnamomi* (**Figure 4D**). As for other systems (Shekhova et al., 2017), H_2O_2 incubation increased the ROS level in both *Phytophthora* spp. In particular, the ROS fluorescence intensity of tagatose-incubated *P. infestans* was intermediate between that of control and H_2O_2-incubated samples. Taken together, these results showed that tagatose decreased the ATP content and OCR with a consequent increase of ROS accumulation in *P. infestans*, but not in *P. cinnamomi*.

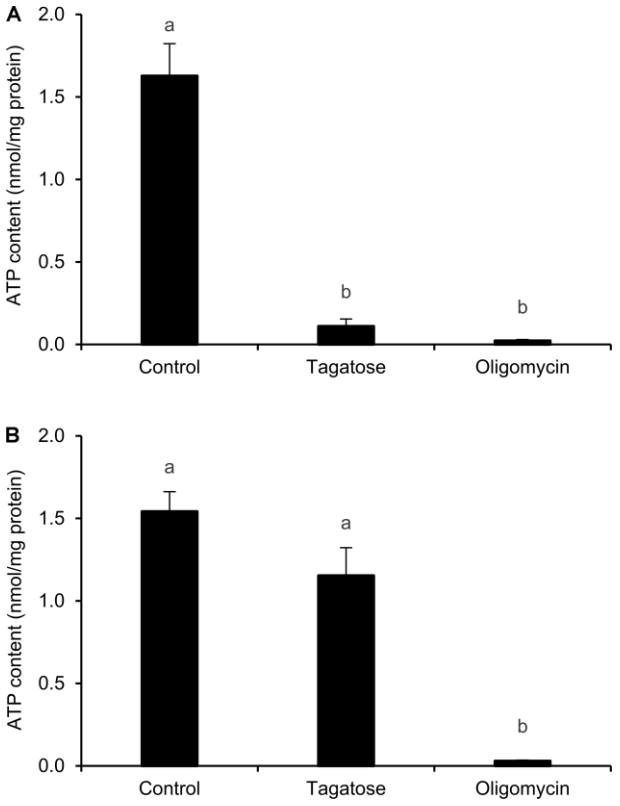

FIGURE 3 | Effect of tagatose on ATP content of *Phytophthora* spp. The ATP content of *P. infestans* (A) and *P. cinnamomi* (B) mycelial suspension was assessed 24 h after incubation in pea broth in the absence (Control) and presence of 5 g/L tagatose or 10 µg/mL oligomycin. The ATP content was quantified using the luminescence assay and expressed per unit of total proteins (nmol/mg). The Kruskal–Wallis test showed no significant differences between the two experimental repetitions ($P > 0.05$, three replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of six replicates (wells) from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments according to the Kruskal–Wallis test ($P \leq 0.05$).

Tagatose Modulates the Expression of *Phytophthora* spp. Genes

Phytophthora spp. genes encoding key enzymes of glycolysis were analyzed by qPCR (**Supplementary Table S1**), such as glucose-6-phosphate dehydrogenase (*g6pd*) and phosphofructokinase (*pfk*). Moreover, genes encoding ATP synthase subunits, responsible for ATP production (Yoshida et al., 2001) were selected, since their activity was previously linked to mitochondrial structure biogenesis in *Saccharomyces cerevisiae* (Paumard et al., 2002; Lefebvre-Legendre et al., 2005), such as the ATP synthase subunit 4 (*atp4*) and ATP synthase subunit beta (*atpB*). Maleylacetoacetate isomerase (*maai*) was analyzed and it shares sequence homology and key domains with glutathione S-transferase genes (*gst*) upregulated by oxidative stresses (Montibus et al., 2015). The apoptosis-inducing factor (*aif*) was analyzed as marker of ROS scavenging (Klein et al., 2002) and mitochondrial cristae regulation (Cheung et al., 2006), since it was upregulated in response to farnesol in *Aspergillus nidulans* (Savoldi et al., 2008). The pro-apoptotic serine protease (*nma111*) and cytochrome c (*cytc*) were selected as upregulated genes in response to graphene oxide (Zhu et al., 2017) and acetic acid in *S. cerevisiae* (Ludovico et al., 2002), respectively. Possible markers of oxidative phosphorylation were selected, such as genes encoding NADH dehydrogenase ubiquinone flavoprotein (*ndufv*) (Kuhn et al., 2015) and cytochrome c oxidase (*cox*) (Dufour et al., 2000), as well as the cellulose synthase (*ces*) responsible for cell wall biosynthesis (Blum et al., 2010).

Expression levels of genes related to apoptosis (*aif* and *nma111*) and oxidative stress response (*maai*) were upregulated by tagatose in *P. infestans* at 4 and 10 DAI (**Table 1**). The *atp4* expression was upregulated by tagatose at 4 DAI, suggestingan attempted *P. infestans* response to the ATP decrease and

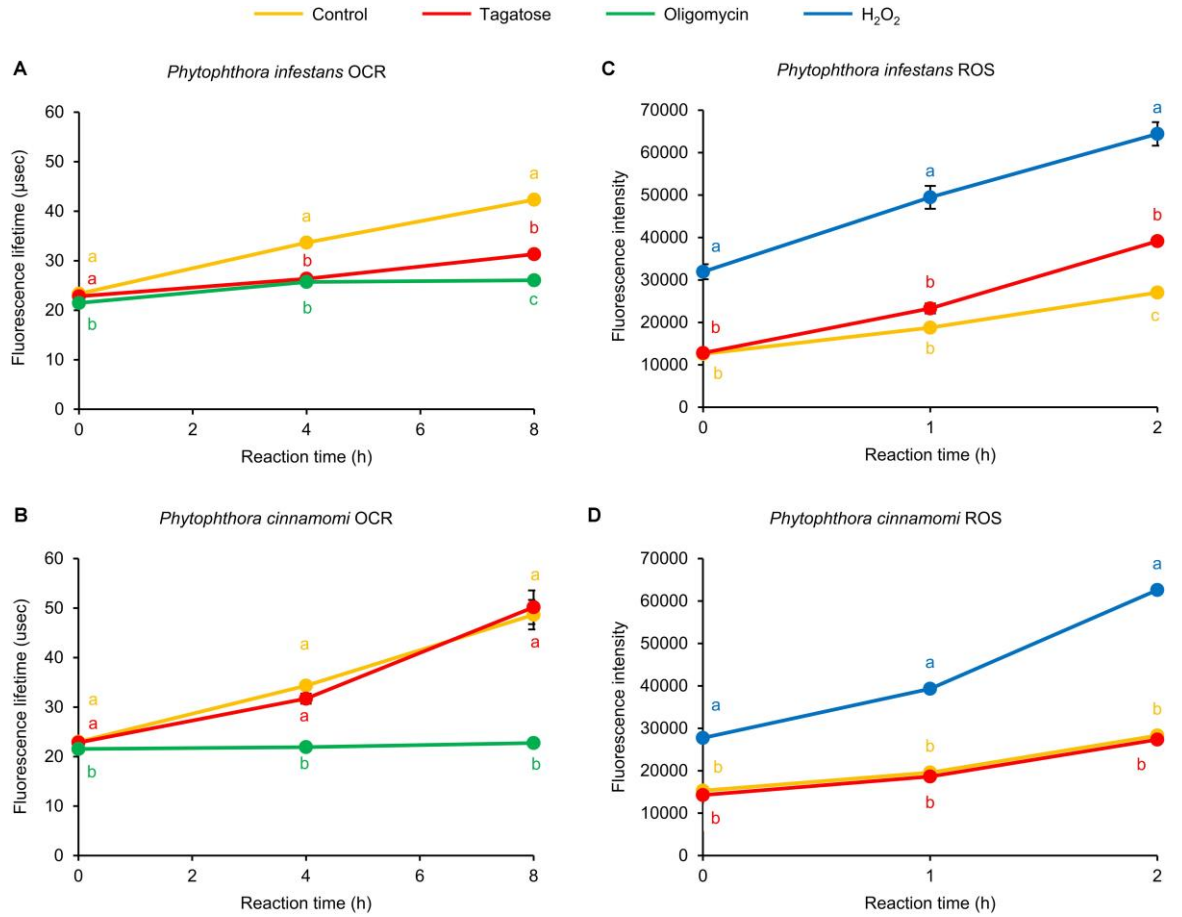

FIGURE 4 | Effect of tagatose on *Phytophthora* spp. respiration processes and accumulation of reactive oxygen species (ROS). The oxygen consumption rate (OCR) and the generation of ROS of the *P. infestans* (A,C) and *P. cinnamomi* (B,D) mycelial suspension were assessed 16 h after incubation in pea broth in the absence (Control, orange) and presence of 5 g/L of tagatose (red). Oligomycin (10 µg/mL, green) and hydrogen peroxide (2 mM H_2O_2, blue) were used as control treatments of the OCR and ROS assay, respectively. The OCR was assessed as increase of fluorescence lifetime (µsec) at 0, 4, and 8 h of reaction time and ROS generation was quantified as fluorescence intensity at 0, 1, and 2 h of reaction time using fluorescent probes. For each assay, the factorial analysis of variance showed no significant differences between the two experimental repetitions ($P > 0.05$, three replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of six replicates (wells) from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments, according to the Tukey test ($P \leq 0.05$).

ROS increase caused by tagatose. On the other hand, the expression of *atpB*, *ces*, *cytc*, *cox*, *g6pd*, *ndufv*, and *pfk* was not affected by tagatose in *P. infestans*. In *P. cinnamomi*, no genes of glycolysis (*g6pd* and *pfk*), ATP synthesis (*atp4* and *atpB*), apoptosis (*aif*), oxidative phosphorylation (*ndufv* and *cox*), cellulose biosynthesis (*ces*), and oxidative stress response (*maai* and *cytc*) were modulated by tagatose at 4 DAI and 10 DAI, except for the *nma111* downregulation at 10 DAI, as corroboration of slight tagatose effects on *P. cinnamomi*.

DISCUSSION

Tagatose is a rare sugar that can be metabolized by onlycertain microbial taxa (Raichand et al., 2012; Martinussen et al., 2013; Van Der Heiden et al., 2013; Wu and Shah, 2017) and inhibits some important crop pathogens, *P. infestans* included (Ohara et al., 2008). Nutritional and anti-nutritional effects of tagatose have been shown on human-associated microorganisms (Van Der Heiden et al., 2013; Lobete et al., 2017; Wu and Shah, 2017; Hasibul et al., 2018) and plant-associated microorganisms (Komon-Zelazowska et al., 2007; Izumori et al., 2008; Ohara et al., 2008; Hayer et al., 2013; Perazzolli et al., 2020). We showed that tagatose inhibited the growth of *P. infestans*, but it had only slight effects on *P. cinnamomi*, with a species-specific impact on the mitochondrial processes. *P. infestans* and *P. cinnamomi* were grown at the respective optimum temperature commonly used for fungicide assays *in vitro* (Coffey and Joseph, 1985; Groves and Ristaino, 2000; Yuan et al., 2006; Hu et al., 2010) and the contribution of temperature to the differential effect of tagatose cannot be totally excluded. The selectivity of action was previously observed on *Trichoderma* spp., where tagatose supported the growth of *T. harzianum* and *T. pleuroticola*, but not that of *T. pleurotum*

TABLE 1 | Effect of tagatose on *Phytophthora* spp. gene expression.

Gene description	Abbreviation	*Phytophthora infestans*		*Phytophthora cinnamomi*	
		4 DAI	10 DAI	4 DAI	10 DAI
Apoptosis inducing factor mitochondria associated	*aif*	1.8 ± 0.1*	1.5 ± 0.1*	1.1 ± 0.1	−1.3 ± 0.3
ATP synthase subunit 4	*atp4*	1.5 ± 0.1*	1.4 ± 0.1	1.1 ± 0.1	1.1 ± 0.0
ATP synthase subunit beta	*atpB*	1.2 ± 0.2	−0.9 ± 0.0	1.0 ± 0.1	1.1 ± 0.1
Cellulose synthase	*ces*	1.3 ± 0.2	1.1 ± 0.0	−1.3 ± 0.1	−1.8 ± 0.1
Cytochrome c	*cytc*	1.0 ± 0.1	−1.2 ± 0.1	1.0 ± 0.2	−1.4 ± 0.3
Cytochrome c oxidase	*cox*	1.4 ± 0.6	1.6 ± 0.6	−1.3 ± 0.1	1.2 ± 0.0
Glucose-6-phosphate dehydrogenase	*g6pd*	1.1 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.4
Maleylacetoacetate isomerase	*maai*	5.8 ± 0.4*	8.3 ± 1.1*	1.1 ± 0.2	−1.2 ± 0.6
NADH dehydrogenase ubiquinone flavoprotein	*ndufv*	−1.1 ± 0.1	1.3 ± 0.1	1.0 ± 0.3	−1.1 ± 0.2
Phosphofructokinase	*pfk*	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.2	−1.3 ± 0.2
Pro-apoptotic serine protease nma111-like protein	*nma111*	2.0 ± 0.3*	2.0 ± 0.2*	−1.3 ± 0.2	−3.2 ± 0.1*

Relative expression levels (fold changes) of P. infestans and P. cinnamomi target genes (Supplementary Table S1) were assessed by quantitative real-time PCR 4 and 10 days after incubation (DAI) on PAM in the absence (Control) and presence of 5 g/L tagatose. The two-way analysis of variance (ANOVA) revealed no significant differences between the two experimental repetitions ($P > 0.05$, five replicates per experiment) and data from the two experiments were pooled. Mean fold change and standard error values of 10 replicates (dishes) from the two experiments are presented for tagatose-incubated samples as compared to the respective control samples, using β-tubulin as a constitutive gene for normalization. Asterisks indicate significant gene upregulation and downregulation in tagatose-incubated samples as comparedto the respective control samples with a fold change greater than 1.5 according to the Student's t-test ($P \leq 0.05$).

(Komon-Zelazowska et al., 2007). Similar selectivity was reported in some bacterial genera, for example tagatose can be assimilated by *Bacillus licheniformis* (Van Der Heiden et al., 2013), *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus brevis* (Bautista et al., 2000), *Lactobacillus casei*, and *L. rhamnosus* (Koh et al., 2013), but not by *B. cereus*, *Bacillus subtilis*, and *Lactobacillus buchneri* (previously called *Lactobacillus frigidus*) (Bautista et al., 2000). Although all the rare sugars testedin this work were epimers (psicose, sorbose, and tagatose), only tagatose inhibited *P. infestans* growth, suggesting that structural differences among epimers may affect the inhibitory activities of rare sugars. The inhibition of *P. infestans* growth was reversible and tagatose-incubated plugs can normally grow when transferred on a new growth medium free of tagatose. This reversible effect raises the question of how to keep the persistence of tagatose on treated crops at constant and sufficient levels, when looking at a possible tagatose application as a plant protection product, and suggest that appropriated formulations should be probably developed for its application under field conditions.

Severe mitochondrial alterations with concentric cristae were found in tagatose-incubated *P. infestans*. Similar alterations have been previously observed in mammalian (Florea and Craciun, 2011) and yeast (Arselin et al., 2004) cells treated with inhibitors of mitochondrial activities, such as *Apis mellifera* venom and doxycycline, respectively. Likewise, some toxic compoundsare known to form mitochondrial concentric cristae in target organisms, such as a phosphocholine derivative in *Leishmania amazonensis* (Godinho et al., 2013), benzimidazole anthelmintic in *Haemonchus contortus* (Cristina et al., 2015), and ethidium bromide in green *Euglena* spp. cells (Nass and Ben-Shaul, 1973). Moreover, xylitol and sorbose caused structural alterations of *Coprinus lagopus* cell wall (Moore and Stewart, 1972), *Streptococcus mutans* cell membrane (Nayak et al., 2014), and *Neurospora crassa* vesicle number and size (Trinci and Collinge, 1973), respectively. Mitochondrial cristae alterations were previously associated with the dysfunction of ATP synthase activity (Paumard et al., 2002; Gavin et al., 2004; Weimann et al., 2008) and with the reduction of mitochondrial bioenergetic status (Zick et al., 2009) in yeast cells. In particular, the formation of concentric cristae was associated with dimerization and oligomerization disorders of the ATP synthase in *S. cerevisiae* (Zick et al., 2009) with the consequent uncontrolled biogenesis of the inner mitochondrial membrane (Velours et al., 2009). The ATP synthase dimerization is associated to disulfide bonds between subunits codified by *atp4* in *S. cerevisiae* (Paumard et al., 2002) and the upregulation of *atp4* in *P. infestans* suggested an attempted cellular response against tagatose, in order to mitigate ATP4 dimerization effects. As consequence of mitochondrial alterations, the ATP content was decreased by tagatose in *P. infestans*, as previously reported in human subjects exposed to tagatose (Buemann et al., 2000) and isolated perfused liver treated with xylitol (Woods and Krebs, 1973). The ATP synthase inhibition has been commonly linked to the OCR reduction (Galloway et al., 2012; Kooragayala et al., 2015) and cellular redox state alteration (Martinez-Reyes and Cuezva, 2014), indicating that OCR inhibition and ROS increase in tagatose-incubated *P. infestans* can be ascribed to severe inhibition of mitochondrial processes.

The attempted cellular responses of *P. infestans* against tagatose included the upregulation of *maai*, which is homologous to *gst* genes commonly upregulated by oxidative stresses (Montibus et al., 2015). The *maai* gene is a marker of stress responses and its expression was also upregulated by the biocontrol agent *Lysobacter capsici* AZ78 in *P. infestans* (Tomada et al., 2017) and by copper sulfate in *Saprolegnia parasitica* (Hu et al., 2016). In addition, tagatose upregulated the expression of *aif* in *P. infestans* and it encoded a protein involved in

ROS scavenging (Klein et al., 2002) and mitochondrial cristae regulation (Cheung et al., 2006) in mammalian cells. The expression of *nma111* was upregulated and downregulated by tagatose in *P. infestans* and *P. cinnamomi*, respectively, and its expression was previously linked to ROS accumulation in *Saccharomyces* spp. (Wang et al., 2014), demonstrating a strong connection of physiological effects and transcriptional changes observed in tagatose-incubated *P. infestans* and *P. cinnamomi*. Therefore, further transcriptomic and metabolomic studies are required, in order to better understand the species-specific response of *Phytophthora* spp. to tagatose incubation and to estimate the risk of evolution of *P. cinnamomi*-like tolerance to tagatose in *P. infestans*.

CONCLUSION

Tagatose inhibited *P. infestans* growth *in vitro* and caused severe ultrastructural alterations, with the formation of circular and concentric mitochondrial cristae. In addition, the ATP content and OCR were decreased, while the ROS accumulation and expression of apoptosis- and oxidative stress-related genes were increased, suggesting the induction of severe deficiencies in the mitochondrial processes of tagatose-incubated *P. infestans*. On the other hand, *P. cinnamomi* growth and mitochondrial ultrastructure were only slightly affected by tagatose with no significant impacts on respiration processes and ROS accumulation, suggesting species-specific responses to this rare sugar. A partial or total selectivity of a fungicide is commonly regarded as a positive trait, because of the reduction of possible side effects on other microorganisms. For this reason, besides the good toxicological profile, tagatose seems to be a promising active substance for the further development of plant protection products to control *P. infestans*. The mode of action of tagatose against *P. infestans* is mainly based on the inhibition of mitochondrial processes, but further transcriptomic and metabolomic analyses are required to fully clarify the molecular determinants and pathways affected by this rare sugar in *Phytophthora* spp. In addition, its reversible effects suggest that efficacy trials of formulated products under field conditions are required, in order to better verify the stability and persistence of tagatose against target phytopathogens.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AC carried out the functional experiments and wrote the manuscript. AN carried out the functional experiments. LN and BB carried out ultrastructural analysis and revised the manuscript. IB carried out growth experiments and an ultrastructural analysis. EA revised the manuscript. IP revised the manuscript and analyzed the data. GP conceived the study, analyzed the data and revised the manuscript. MP conceived the study, supervised the experiments, analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00128/full#supplementary-material

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Publication 2: A review of knowledge on the mechanisms of action of the rare sugar D-tagatose against phytopathogenic oomycetes (submitted to Plant Pathology)

Résumé: Le D-tagatose est un sucre rare, naturellement présent à de faibles concentrations dans certains fruits et produits laitiers et utilisé comme édulcorant dans l'industrie alimentaire car il ne présente aucun danger pour la santé humaine. Le D-tagatose est capable d'inhiber la croissance de nombreux micro-organismes, tels que les oomycètes phytopathogènes responsables d'importantes maladies des cultures. Grâce à ses effets négligeables sur la santé humaine et l'environnement, le D-tagatose a été proposé comme un produit durable pour la protection des cultures. Cette revue décrit les connaissances actuelles sur les modes d'action du D-tagatose contre les oomycètes phytopathogènes et ses utilisations potentielles en agriculture. Le D-tagatose peut affecter négativement la croissance des oomycètes phytopathogènes en inhibant des enzymes du métabolisme des glucides, comme la β-glucosidase chez *Phytophthora infestans*, la fructokinase et la phosphomannose isomérase chez *Hyaloperonospora arabidopsidis*. De plus, le D-tagatose provoque de graves altérations mitochondriales et inhibe les processus de respiration avec l'accumulation d'espèces réactives de l'oxygène et l'induction des gènes liés à l'apoptose et au stress oxydatif chez *P. infestans*, mais pas chez *P. cinnamomi*. Les configurations structurelles des sucres rares peuvent être responsables de l'inhibition compétitive des enzymes clés du métabolisme des glucides, mais des études biochimiques supplémentaires sont nécessaires pour vérifier les interactions des sucres rares avec les sites catalytiques. Le D-tagatose n'agit pas comme un éliciteur de résistance des plantes, contrairement à d'autres sucres rares, tels que le D-psicose et le D-allose. Le D-tagatose permet de lutter contre les maladies par pulvérisation foliaire, par arrosage du sol ou par traitement des semences, et aucun effet phytotoxique n'a été observé sur les plantes traitées. De plus, le D-tagatose a des effets à la fois préventifs et curatifs contre certaines maladies des plantes. Des essais en serre et en plein champ ont montré que l'efficacité du D-tagatose contre le mildiou de la vigne, du concombre, du chou chinois, de l'oignon et de l'épinard est comparable à celle obtenue avec des fongicides chimiques (par exemple le chlorothalonil, le cuivre, le cyazofamid, le métalaxyl et le mancozèbe). Ce résultat suggère un grand potentiel de ce sucre rare pour remplacer les fongicides chimiques dans la protection des cultures.

A review of knowledge on the mechanisms of action of the rare sugar D-tagatose againstphytopathogenic oomycetes

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Running title: D-tagatose against phytopathogenic oomycetes

Abstract

D-tagatose is a rare monosaccharide, naturally present at low concentrations in some fruits and dairy products. The D-tagatose is generally recognized as safe and it is used as low-calorie sweetener in the food industry. D-tagatose is able to inhibit the growth of numerous microorganisms, such as phytopathogenic oomycetes responsible for important crop diseases. Thanks to the negligible effects on human health and the environment, D-tagatose was proposed as a sustainable product for crop protection. This review describes the current knowledge on modes of action D-tagatose against phytopathogenic oomycetes and its potential uses in agriculture. D-tagatose can negatively affect the growth of phytopathogenic oomycetes by inhibiting key enzymes of the sugar metabolism, such as β-glucosidase in *Phytophthora infestans*, fructokinase and phosphomannose isomerase in *Hyaloperonospora arabidopsidis*. Moreover, the D-tagatose causes severe mitochondrial alterations and inhibits respiration processes with the accumulation of reactive oxygen species and the upregulation of apoptosis- and oxidative stress-related genes in *P. infestans*, but not in *P. cinnamomi*. Thus, D-tagatose displays species-specific effects on *Phytophthora* spp. and selective nutritional properties on some plant-associated microorganisms. However, inhibitory effects are reversible and *P. infestans* growth can be restored in the absence of D-tagatose. Further functional studies are therefore required, in order to improve D-tagatose efficacy and to understand mechanisms responsible for species-specificity.

KEYWORDS

Rare sugar, tagatose, microbial growth inhibition, phytopathogenic oomycetes, mechanism of action

1. **INTRODUCTION**

Rare sugars are defined as monosaccharides that exist only in a small amount in nature (Izumoriet al., 2008). Rare sugars comprise twenty hexoses (D-allose, D-altrose, D-gulose, D-idose, D- talose, L-allose, L-altrose, L-glucose, L-mannose, L-gulose, L-idose, L-galactose, L-talose, D-psicose, D-sorbose, D-tagatose, L-fructose, L-psicose, L-sorbose and L-tagatose) and nine pentoses (D-arabinose, D-lyxose, L-ribose, L-xylose, L-Lyxose, D-ribulose, D-xylulose, L- ribulose and L-xylulose) and most of them are isomers of the seven common monosaccharides that exist in large amount in nature, such as D-glucose, D-fructose, D-galactose, D-mannose, D-ribose, D-xylose and L-arabinose (Ahmed, 2001, Izumori et al., 2008, Jayamuthunagai et al.,2017). The biological properties of rare sugars are not fully understood and their promising applicative values are underestimated, mainly because of their limited available quantity in nature (Li et al., 2013). However, the development of innovative enzymatic and microbial methods for rare sugar synthesis lowered the cost of production and expanded their application in several scientific and technological areas of medicine, food and agriculture (Granström et al., 2004, Oh, 2007, Li et al., 2013).

Among rare sugars, D-tagatose is naturally present at low concentrations in foods, such asapple, orange, milk and cheese (Vastenavond et al., 2011). D-tagatose is also present as a metabolic intermediate of the tagatose-6-phosphate pathway, which is activated for the degradation of D-galactose and D-lactose in some bacteria, such as *Staphylococcus aureus* and *Streptococcus lactis* (Bissett and Anderson, 1974). D-tagatose is an epimer of D-fructose, withan inversion of the spatial configuration of hydroxyl group of the fourth carbon (C4-OH group; Figure 1).

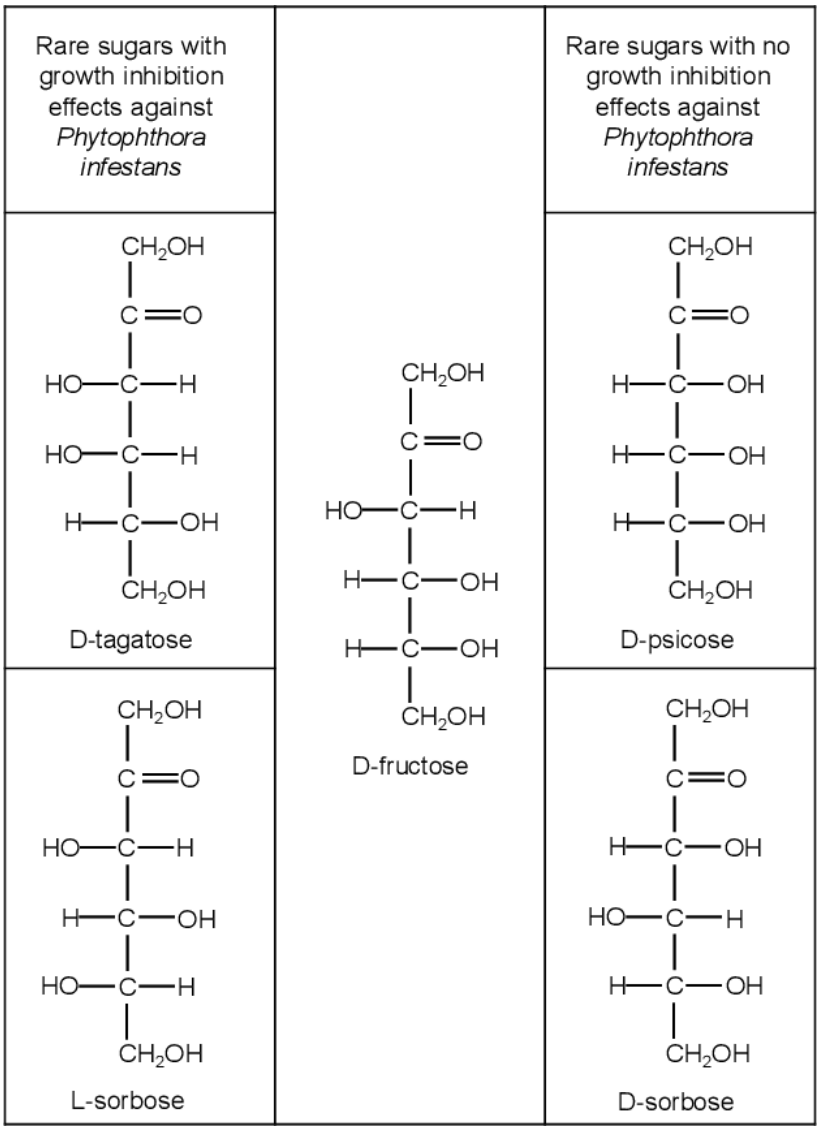

Figure 1. Fisher projections of some rare sugars. Fisher projections and inhibitory properties on *Phytophthora infestans* are reported for some rare sugars with structural similarities with D-fructose and potential applicative value. According to the protocol reported by Chahed et al. (2020), *P. infestans* growth was inhibited ($P \leq 0.05$; Kruskal-Wallis test) by 5 g/L D-tagatose (84.20 ± 0.23%%; mean inhibition percentage and standard error compared to control samples) (Chahed et al., 2020) and 5 g/L L-sorbose (42.04 ± 0.12 this study), but it was not affected by 5 g/L D-psicose (0.22 ± 0.13 %), 5 g/L D-sorbose (0.39 ± 0.15 %) and 5 g/L D-fructose (0.19 ± 0.11 %) (Chahed et al., 2020).

D-tagatose can be synthesized from D-lactose in a two-step process, where D-lactose is enzymatically hydrolysed to D-glucose and D-galactose using lactase in the first step (Figure

2) (Bertelsen et al., 1999). In the second step of the chemical synthesis, D-galactose is then isomerized to D-tagatose in the presence of calcium hydroxide (Bertelsen et al., 1999). Although the chemical synthesis is an efficient method for the large-scale production of D-tagatose, consumer concerns and safety issues stimulated the development of processes based on biological synthesis (Roy et al., 2018). In particular, bacterial L-arabinose isomerase can be used for the isomerisation of D-galactose to D-tagatose in the second step of the biological synthesis (Figure 2) (Kim, 2004).

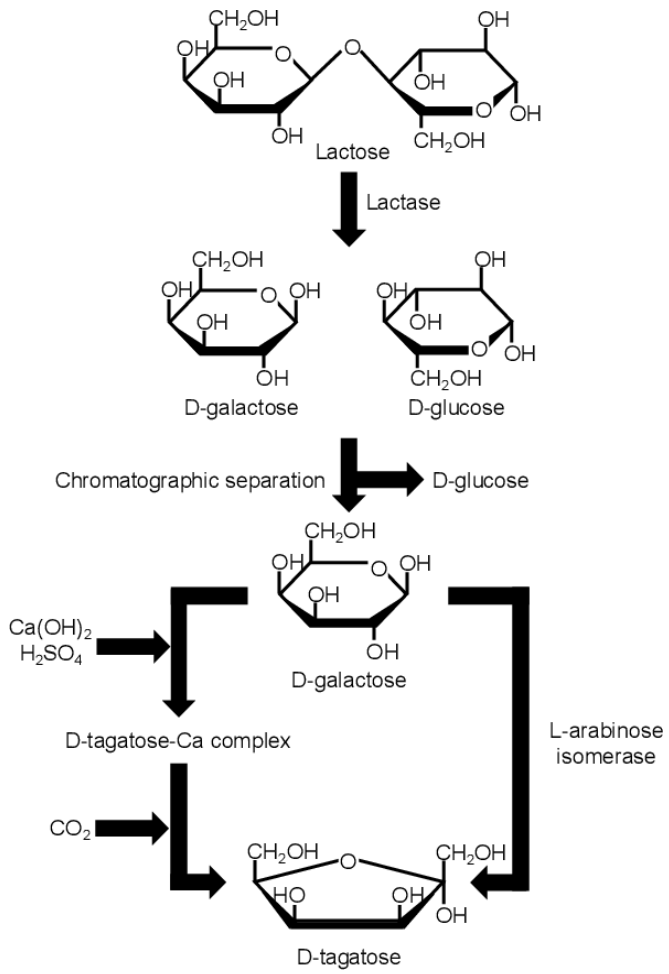

Figure2. D-tagatose chemical and biological synthesis reactions. D-tagatose synthesis starts with the hydrolysis of lactose into D-glucose and D-galactose by lactase enzyme. In the chemical synthesis, D-galactose is then isomerized to D-tagatose with calcium hydroxide [$Ca(OH)_2$] and the reaction is stopped by adding sulfuric acid

(H_2SO_4). The chemical process requires an additional reaction using carbon dioxide (CO_2). In the biological synthesis, D-galactose is isomerized to D-tagatose by L-arabinose isomerase. Synthesis reactions are summarised according to Bertelsen et al. (1999) and Kim (2004).

D-tagatose was generally recognized as safe by the Food and Drug Administration in US, because it has no negative impact on human health (Levin, 2002, Vastenavond et al., 2011). Thanks to its low caloric content (1.5 kcal/g) and small glycemic index compared to sucrose, D-tagatose is currently used in the food industry as a low calorie sweetener (Vastenavond et al.,2011). In addition, D-tagatose has shown therapeutic properties on human subjects and has been proposed to control type 2 diabetes, hyperglycaemia, anaemia, haemophilia and obesity (Levin, 2002). D-tagatose affects the growth of some human-associated microorganisms and has prebiotic properties on the human gut microbiota, by increasing the abundance of beneficial bacteria (e.g. *Enterococcus* spp. and *Lactobacillus* spp.) (Bertelsen et al., 1999, Vastenavond etal., 2011, Hasibul et al., 2018) and inhibiting the growth of some human pathogens (e.g. *Streptococcus mutants* and *Salmonella enterica* serovar Typhimurium) (Lobete et al., 2017, Hasibul et al., 2018). D-tagatose can be used as a carbon source by only few microbial taxa, such as *Exiguobacterium* spp., *Lactobacillus* spp. and *Lactococcus* spp. (Raichand et al., 2012,Martinussen et al., 2013, Van der Heiden et al., 2013, Wu and Shah, 2017). In particular, the D-tagatose metabolism involves the tagatose-6-phosphate pathway in *Lactobacillus* spp. and *Lactococcus* spp. (Martinussen et al., 2013, Wu and Shah, 2017). Moreover, D-tagatose incubation causes a complex transcriptional reprogramming of the carbohydrate metabolism in *L. rhamnosus* (Koh et al., 2013), indicating precise metabolic adaptations for the rare sugar catabolism in bacteria. On the other hand, D-tagatose is not catabolised by several human pathogenic bacteria, such as *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *S. enterica* serovar Typhimurium, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Yersiniaenterocolitica* (Bautista et al.,

2000), indicating anti-nutritional properties on specific microbialtaxa. Likewise, D-tagatose can selectively inhibit the growth of some phytopathogens (Chahedet al., 2020, Mochizuki et al., 2020, Perazzolli et al., 2020). The aim of this review is to summarise the current knowledge on the modes of action of D-tagatose against phytopathogenicoomycetes and on the possible use of this rare sugar for sustainable crop protection.

2. D-TAGATOSE INHIBITS THE GROWTH OF A WIDE RANGE OF PLANT PATHOGENS

D-tagatose inhibits the growth of a wide range of phytopathogens with negligible effects on human health and the environment, therefore its efficacy was tested against several crop diseases (Ohara et al., 2008). For instance, D-tagatose controls grapevine powdery mildew (*Erysiphe necato*r), tomato grey mould (*Botrytis cinerea*), alternaria sooty spot (*Alternaria brassicicola*), brown rust (*Puccinia recondita*) and rice sheath blight (*Rhizoctonia solani*) (Mochizuki et al., 2020, Perazzolli et al., 2020). Among phytopathogens, several oomycetes are inhibited by D-tagatose, such as *Hyaloperonospora arabidopsidis, H. parasitica, Peronospora destructor, P. farinosa, Phytophthora infestans*, *Plasmopara viticola*, *Pseudoperonospora cubensis*, *Pythium aphanidermatum* and *P. graminicola* (Chahed et al., 2020, Mochizukiet al., 2020, Perazzolli et al., 2020, Corneo et al., 2021). Disease control is achieved by D- tagatose foliar spray, soil drenching or seed treatment and no phytotoxic effects are noticed on treated plants (Ohara et al., 2008). In addition, D-tagatose has both preventive and curative effects against some plant diseases, such as cucumber downy mildew caused by *P. cubensis* (Mochizuki et al., 2020). In particular, D-tagatose treatment inhibits downy mildew development when cucumber plants are treated five days before or two days after pathogen inoculation (Mochizuki et al., 2020). Greenhouse and field trials showed that D-tagatose efficacy against downy mildew of grapevine, cucumber, Chinese cabbage, onion

and spinach is comparable to that obtained with chemical fungicides (e.g. chlorothalonil, copper, cyazofamid, metalaxyl and mancozeb) (Mochizuki et al., 2020, Perazzolli et al., 2020), suggesting a great potential of this rare sugar to substitute synthetic chemical fungicides in crop protection.

D-tagatose has a double effect on plant health by acting as an anti-nutritional molecule on some phytopathogens (direct effect) and as a nutritional factor on some indigenous biocontrol microorganisms (indirect effect) (Perazzolli et al., 2020). In particular, D-tagatose treatments of grapevine decreases the relative abundance of *Erysiphe* spp., in agreement with the reduction of powdery mildew symptoms caused by *Erysiphe necator* (Perazzolli et al., 2020). On the other hand, D-tagatose exhibits a prebiotic effect on the phyllosphere microbiota and increases the relative abundance of some natural microorganisms with potential beneficial effects on grapevine (e.g. *Alternaria* spp., *Aureobasidium* spp., *Exiguobacterium* spp. and *Exophiala* spp.) that could partially contribute to disease control by competing for space and nutrients or by inducing plant resistance (Perazzolli et al., 2020).

3. D-TAGATOSE HAS VARIOUS MECHANISM OF ACTION AGAINST PHYTOPATHOGENIC OOMYCETES

The growth inhibition of phytopathogenic oomycetes caused by D-tagatose is associated with negative impacts on multiple metabolic processes (Figure 3) (Chahed et al., 2020, Mochizuki et al., 2020, Corneo et al., 2021).

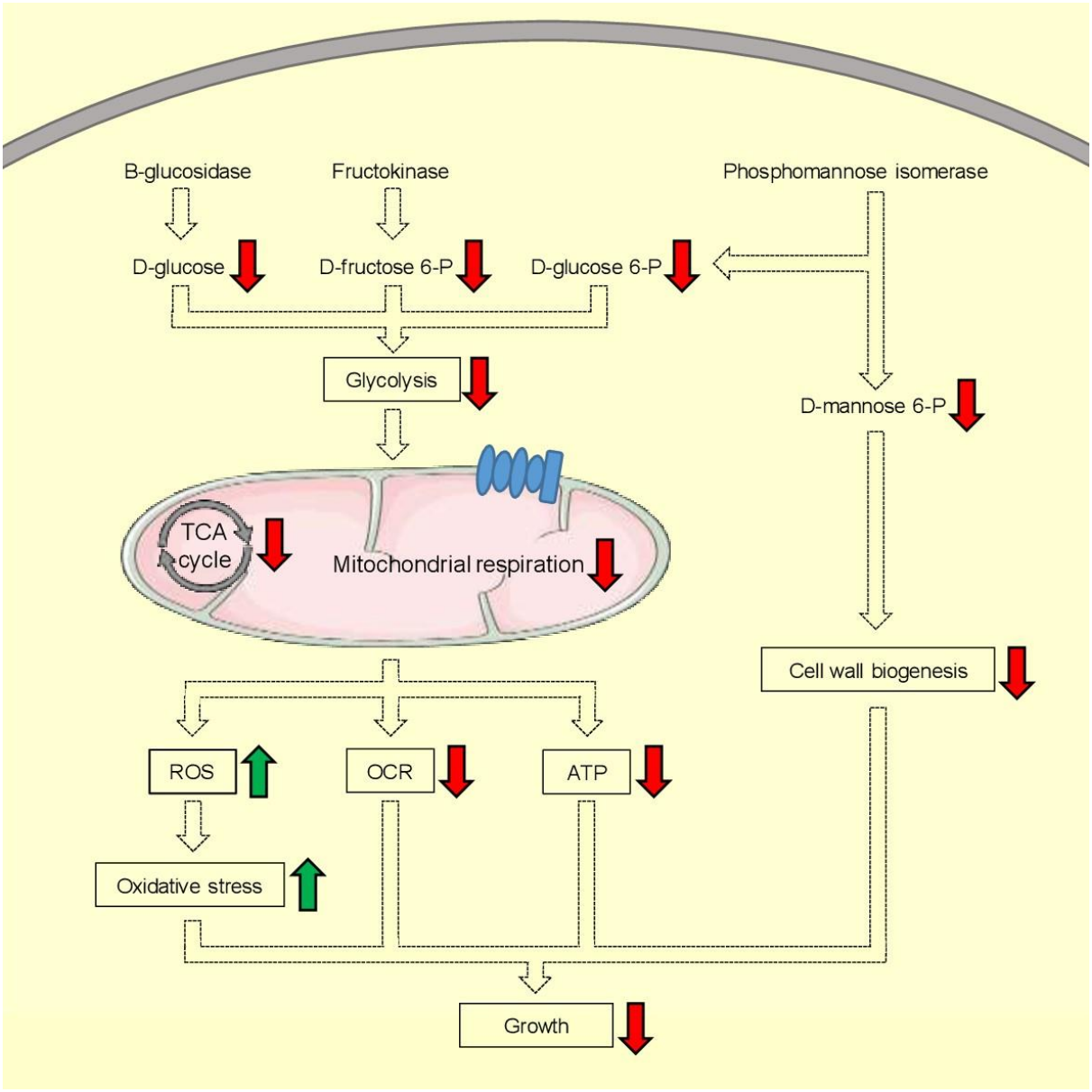

Figure 3: Possible mechanisms of action of D-tagatose against phytopathogenic oomycetes. D-tagatose incubation inhibits β-glucosidase, fructokinase and phosphomannose isomerase activity, decreases D-glucose, D- fructose 6-phosphate (D-fructose 6-P), D-glucose 6-P and D-mannose 6-P content, leading to glycolysis inhibition, tricarboxylic acid (TCA) cycle inhibition and cell wall biogenesis deficiency. In addition, D-tagatose causes severe mitochondrial alterations, with the consequent decrease in ATP content, reduction in oxygen consumption rate (OCR), accumulation of reactive oxygen species (ROS) and increase of oxidative stress. Increases (green arrow) or decreases (red arrow) in metabolic processes and intermediates are visualised according to D-tagatose effects on phytopathogenic oomycetes (Chahed et al., 2020, Mochizuki et al., 2020, Corneo et al., 2021) using Servier Medical Art templates (https://smart.servier.com).

In particular, D-tagatose causes severe mitochondrial alterations in *P. infestans*, with the consequent decrease in ATP content, oxygen consumption rate and mycelial growth (Chahed et al., 2020). Moreover, the incubation of *P. infestans* with D-tagatose causes the accumulation of reactive oxygen species (ROS) and the upregulation genes related to apoptosis and oxidative stress response (Chahed et al., 2020), indicating possible oxidative damages on cell structures. Likewise, the rare sugar L-sorbose affects the growth and morphology of *Neurospora crassa*, with a general metabolic perturbation derived from a partial uncoupling of respiration and oxidative phosphorylation (Crocken and Tatum, 1968). Mitochondrial alterations in D- tagatose-incubated *P. infestans* could be associated with dysfunction of ATP synthase activity, as found in yeast cells (Paumard et al., 2002, Gavin et al., 2004, Weimann et al., 2008), and with decreased mitochondrial bioenergetic status and ATP content, as found in human subjects exposed to D-tagatose (Buemann et al., 2000).

The alterations of mitochondrial processes found in D-tagatose-incubated *P. infestans* (Chahed et al., 2020) are associated with sugar metabolism inhibition in oomycetes (Mochizukiet al., 2020, Corneo et al., 2021). In particular, *H. arabidopsidis* growth inhibition caused by D-tagatose is ascribed to a competitive inhibition of fructokinase and phosphomannose isomerase enzymes (Mochizuki et al., 2020). Since fructokinase can phosphorylate both D- fructose and D-tagatose, the incubation of *H. arabidopsidis* with D-tagatose decreases D- fructose 6-phosphate content and increases D-tagatose 6-phosphate content (Mochizuki et al., 2020). Consequently, D-tagatose 6-phosphate inhibits phosphomannose isomerase and leads to a decrease in D-mannose 6-phosphate and D-glucose 6-phosphate production (Mochizuki et al.,2020). Thus, D-tagatose decreases the content of D-fructose 6-phosphate and D-glucose 6- phosphate, which are key intermediates of glycolytic pathways, and the content of D-mannose 6-phosphate, which is required for cell wall synthesis (Mochizuki et al., 2020). The cell wall of oomycetes is mainly composed of

D-glucose and D-mannose polymers (Melida et al., 2013), and phosphomannose isomerase inhibition may be responsible for cell wall biogenesis deficiency and growth inhibition (Mochizuki et al., 2020). Likewise, D-tagatose is reported as a glycolysis inhibitor in human cells (Kim et al., 2014) and shows inhibitory effects on fructose phosphate aldolase in *E. coli* (Stellmacher et al., 2016), fructokinase (Lu et al., 2008) and glucosidase (Espinosa and Fogelfeld, 2010) in mammals. In agreement with the inhibition of metabolic reactions, D-tagatose decreases β-glucosidase activity, D-glucose, D-glucose 1- phosphate and mannose content, while it increases D-fructose, D-galactose and D-sucrose content in *P. infestans* (Corneo et al., 2021). As a possible consequence of glycolysis inhibition, the content of tricarboxylic acid cycle intermediates (e.g. malic acid, succinic acid and α- ketoglutaric acid) is decreased by the incubation of *P. infestans* with D-tagatose (Corneo et al.,2021). Inhibitory effects on microbial enzymes can also be caused by other rare sugars, such as L-sorbose 1-phosphate and L-sorbose 1,6-bisphosphate on yeast aldolase (Richards and Rutter,1961) and D-xylitol on *S. mutants* glycolysis (Assev and Rolla, 1986), suggesting antagonistic interactions between rare sugar and common sugar metabolism. The existence of antagonistic interactions between rare sugars and common sugars was supported by the attenuation of D-tagatose effects on *P. infestans* (Corneo et al., 2021) and *S. mutants* (Hasibul et al., 2018) growth in the presence of D-fructose, but not D-glucose. In particular, D-tagatose-mediated growth inhibition and metabolite changes can be fully impaired by the presence of D-fructose, while they are only partially attenuated by the presence of D-glucose and not influenced by the presence of D-sucrose in *P. infestans* (Corneo et al., 2021). Thanks to their similar chemical structure, D-tagatose and D-fructose can serve as substrates for the same enzymes and lead to competition for catalytic sites (Mochizuki et al., 2020). Previous studies showed that the hydroxyl group of the third carbon (C3-OH group) is implicated in the binding of D-fructose tofructose-metabolising enzymes (Bertrand et al., 1998, Nocek et al.,

2011) and in the correct orientation of glucosidase inhibitors (e.g. flavonoids) in the binding pocket of the target enzyme (Xu, 2010). Among all tested rare sugars, those with a similar C3 spatial configuration of D-fructose and with a different configuration of the fourth and fifth carbon inhibit *P. infestans* growth (i.e. D-tagatose and L-sorbose), while those with a different C3 spatial configuration do not (i.e. D-psicose and D-sorbose; Figure 1). Thus, structural configurations of rare sugars may be responsible for competitive inhibition of key enzymes of the sugar metabolism, but further biochemical studies are required to verify interactions of rare sugars with catalytic sites.

Although D-tagatose inhibits key enzymes of sugar metabolism (i.e. fructokinase, phosphomannose isomerase and β-glucosidase) in *P. infestans* and *H. arabidopsidis* (Chahed et al., 2020, Mochizuki et al., 2020, Corneo et al., 2021), species-specific effects are observed on phytopathogenic oomycetes (Chahed et al., 2020). In particular, *P. cinnamomi* growth and mitochondrial ultrastructure are only slightly affected by D-tagatose with no impacts on ATP content, respiration processes and ROS accumulation (Chahed et al., 2020). Selectivity of a fungicide is considered as a positive trait, due to the limited side effects on other microorganisms. However, the species-specific effects of D-tagatose on *Phytophthora* spp. raise the question whether species inhibited by this rare sugar can further acquire resistance traits and evolve metabolic adaptations. Further functional and molecular studies are therefore needed to clarify the differential inhibitory mechanisms of D-tagatose and the species-specific responses of oomycetes to this rare sugar. Moreover, the growth inhibition of *P. infestans* is reversible and mycelial growth can be restored on a D-tagatose-free growth medium, suggesting that efficacy trials of formulated products are needed in order to better verify the stability and persistence of D-tagatose under field conditions.

4. **D-TAGATOSE DOES NOT ACT AS A PLANT RESISTANCE INDUCER**

D-tagatose does not act as a plant resistance inducer (Mochizuki et al., 2020) unlike other rare sugars, such as D-psicose and D-allose (Kano et al., 2011). In particular, D-tagatose does not modulate the expression of defence-related genes in cucumber, rice and *Arabidopsis thaliana* (Mochizuki et al., 2020). For example, the transcriptome analysis of *A. thaliana* does not show any significant modulation of hormone- and defence-related genes (e.g. pathogenesis-related proteins, peroxidase, lipoxygenase and pore-forming toxin like protein) in response to D- tagatose treatment and *H. arabidopsidis* inoculation (Mochizuki et al., 2020). Thus, downy mildew suppression by D-tagatose is mainly related to direct inhibition of *H. arabidopsidis* growth rather than induction of *A. thaliana* resistance (Mochizuki et al., 2020). Conversely, D-psicose and D-allose upregulates the expression of defence-related genes (e.g. lipoxygenase L-2, β-1,3-glucanase, peroxidase and chitinase) in rice, resulting in enhanced plant resistance against *Xanthomonas oryzae* (Kano et al., 2011), therefore further studies are required to clarify the effect of rare sugars on plant resistance induction. The different effect of D-psicose and D-tagatose in plant protection suggests that slight structural differences of rare sugars may strongly affect their functional properties. In particular, D-psicose induces rice resistance against *X. oryzae* (Kano et al., 2011), but it does not inhibit *P. infestans* growth (Chahed et al.,2020), while D-tagatose does not induce rice resistance (Mochizuki et al., 2020), but inhibits *P.infestans* growth (Chahed et al., 2020). Thus, the combination of D-tagatose with D-psicose in crop protection strategies could complement their effects on direct inhibition of phytopathogen growth and plant resistance activation.

5. **CONCLUSION**

D-tagatose inhibits the growth of phytopathogenic oomycetes and was proposed as a promising plant protection product with no negative effects on human health and the environment. Studies performed so far showed that the mode of action of D-tagatose against

phytopathogenic oomycetes is mainly based on the inhibition of sugar metabolism and mitochondrial processes. The growth inhibition of phytopathogenic oomycetes can be ascribed to structural similarities of D-tagatose with D-fructose and to competitive inhibition of key metabolic enzymes. However, D-tagatose shows species-specific effects on *Phytophthora* spp. and selective nutritional properties on some beneficial plant-associated microorganisms. Although the specificity of plant protection products may be associated with low side effects on non-target microorganisms, further functional studies are required to understand the differential mechanisms of D-tagatose on phytopathogens and the species-specific microbial responses to this rare sugar.

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CONFLICT OF INTERESTS

AC and AN were employed by Bi-PA nv. The remaining authors declare that the research wasconducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publication 3: The differential growth inhibition of *Phytophthora* spp. caused by the rare sugar tagatose is associated with species-specific metabolic and transcriptional changes (submitted to mSystems)

Résumé: Le tagatose a inhibé la croissance mycélienne et a affecté négativement *Phytophthora infestans*, mais pas celle de *P. cinnamomi*, indiquant des réponses spécifiques à ce sucre rare chez deux espèces du même genre. *Phytophthora infestans* et *P. cinnamomi* ont activé des changements métaboliques et transcriptionnels particuliers en réponse à l'incubation avec ce sucre rare. En particulier, la teneur en sucres et en acides aminés a été modifiée par le tagatose chez *P. infestans*, mais pas chez *P. cinnamomi*. De plus, un nombre élevé de gènes a été modulé chez *P. infestans* (3915 gènes) par rapport à *P. cinnamomi* (512 gènes) suite à l'incubation avec le tagatose, ce qui suggère que cette incubation a provoqué une reprogrammation transcriptionnelle sévère chez *P. infestans*, plutôt que chez *P. cinnamomi*. La reprogrammation transcriptionnelle de *P. infestans* a été principalement caractérisée par une sévère répression des gènes impliqués dans le transport des sucres et des acides aminés, le métabolisme carboné, la signalisation cellulaire et les processus liés à la croissance. A l'inverse, la réponse de *P. cinnamomi* à l'incubation avec le tagatose a été caractérisée par l'activation de processus liés au transport des sucres, au métabolisme énergétique, au métabolisme des glucides et au stress oxydatif pour limiter les impacts négatifs sur la croissance mycélienne, la teneur en sucre et en acides aminés. En particulier, *P. cinnamomi* a été capable de mettre en œuvre de multiples voies d'adaptation pour moduler le métabolisme cellulaire en se basant sur l'activation de gènes liés au transport du glucose, au métabolisme des pentoses, au cycle de l'acide tricarboxylique, à la détoxification des espèces réactives de l'oxygène, à la respiration mitochondriale et à la respiration alternative. Ces résultats représentent une majeure contribution à la caractérisation du mode d'action du tagatose contre deux espèces différentes du genre *Phytophthora*.

The differential growth inhibition of *Phytophthora* spp. caused by the rare sugar tagatose is associated with species-specific metabolic and transcriptional changes

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Running title: Tagatose affect *Phytophthora* spp. metabolism

Abstract

Tagatose is a rare sugar with limited availability in nature and no negative impacts on human health. Tagatose showed selective inhibitory effects on human- and plant-associated microorganisms, phytopathogens included. In particular, tagatose inhibited mycelial growth and negatively affected mitochondrial processes in *Phytophthora infestans*, but not in *P. cinnamomi*, indicating species-specific responses. The aim of this study was to elucidate metabolic changes and transcriptional reprogramming activated by *P. infestans* and *P. cinnamomi* in response to tagatose, in order to clarify the differential inhibitory mechanisms of tagatose and the species-specific reactions to this rare sugar. *Phytophthora infestans* and *P. cinnamomi* activated distinct metabolic and transcriptional changes in response to the rare sugar. In particular, tagatose negatively affected mycelial growth, sugar content and amino acid content in *P. infestans* with a severe transcriptional reprogramming that included the downregulation of genes involved in transport, sugar metabolism, signal transduction and growth-related process. On the other hand, tagatose incubation upregulated genes related to transport, energy metabolism, sugar metabolism and oxidative stress in *P. cinnamomi* with no negative effects on mycelial growth, sugar content and amino acid content. The differential inhibitory effects of tagatose on *Phytophthora* spp. were associated with an attempted reaction of *P. infestans*, which was not sufficient to attenuate the negative impacts of the rare sugar. Conversely *P. cinnamomi* was able to activate an efficient response, which included the reprogramming of multiple metabolic processes, such as genes related to glucose transport, pentose metabolism, tricarboxylic acid cycle, reactive oxygen species detoxification, mitochondrial and alternative respiration processes. Knowledge on the differential response of *Phytophthora* spp. to tagatose represent a step forward in the understanding of functional effects and ecological roles of rare sugars on the microbial metabolism.

KEYWORDS

rare sugar, *Phytophtho*ra spp., transcriptomics, gene expression level, targeted metabolomics,transcriptional reprogramming

Take Aways

1. *Phytophthora infestans* and *P. cinnamomi* activate species-specific responses to tagatose.
2. Tagatose affects growth, sugar and amino acid metabolism in *P. infestans*, but not in *P. cinnamomi*
3. *Phytophthora cinnamomi* response to tagatose includes efficient responses of energy and sugar metabolism
4. Differential responses of *Phytophthora* spp. indicate species-specific ecological roles of rare sugars.

1 | INTRODUCTION

Rare sugars have been defined as monosaccharides and their derivatives that rarely exist in nature, such as tagatose, allose, sorbose, xylulose and xylitol (Granström, Takata, Tokuda, & Izumori, 2004). The potential functional and ecological properties of rare sugars are underestimated due to their limited availability in nature (Li, Gao, Nakanishi, Gao, & Cai, 2013). However, the implementation of novel enzymatic and microbial processes lowered the cost of rare sugar synthesis and extended their use in various industrial and scientific fields, such as agriculture, human nutrition and medicine (Granström etal., 2004; Izumori, 2006; Li et al., 2013). Among rare sugars, tagatose is a ketohexose that was found at low concentrations in many foods, such as apple, pineapple, orange and milk (Vastenavond et al., 2011). Since tagatose does not have negative impacts on human health, it was 'generally recognized as safe' by the Food and Drug Administration and it is used as low-calorie sweetener in several countries (Levin, 2002; Vastenavond et al., 2011). Tagatose can be utilised as a carbohydrate source by only a restricted number of microorganisms, such as *Exiguobacterium* spp., *Lactobacillus* spp. and *Lactococcus* spp. (Martinussen, Solem, Holm, & Jensen, 2013; Raichand, Pareek, Singh, & Mayilraj, 2012; Van der Heiden et al., 2013; Wu & Shah, 2017). On the other hand, tagatose is not catabolised by some human- associated microorganisms, such as *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Staphylococcus aureus* and *Yersinia enterocolitica* (Bautista, Pegg, & Shand, 2000), indicating selective nutritional or anti-nutritional properties for specific microbial taxa. For example, tagatose inhibited the growth of *Streptococcus mutans* (Hasibul et al., 2018) and *Salmonella enterica* serovar Typhimurium (Lobete et al., 2017) and decreased the gene expression (e.g. glucosyltransferase, fructosidase and phosphotransferase encoding genes) and the enzymatic activity (e.g. glucosyltransferase) of sugar metabolism in *S. mutans* (Hasibul et

al., 2018). Conversely, tagatose enhanced the growth of *Lactobacillus rhamnosus* and upregulated genes associated with sugar metabolism (e.g. phosphotransferase encoding genes, phosphofructokinase and tagatose-6-phosphate kinase) (Koh et al., 2013), suggesting specific impacts of rare sugars on the microbial metabolism.

Tagatose inhibited the growth of several causal agents of plant diseases, such as *Phytophthora infestans* (tomato and potato late blight) (Chahed et al., 2020), *Plasmopara viticola* (grapevine downy mildew) and *Erysiphe necator* (grapevine powdery mildew) (Perazzolli et al., 2020), *Hyaloperonospora parasitica* (cabbage downy mildew) and *Oidium violae* (tomato powdery mildew) (Mochizuki et al., 2020). Tagatose showed also possible prebiotic effects on the phyllosphere microbiota and shifted the proportions of potential beneficial and potential pathogenic microorganisms by a selective nutritional or anti-nutritional effect on plant-associated microorganisms (Perazzolli et al., 2020). Differential effects were also found on *Trichoderma* spp. growth, where tagatose supported the growth of *T. harzianum* and *T. pleuroticola*, but not that of *T. pleurotum* (Komon-Zelazowska et al., 2007), confirming the selective effect of tagatose on plant-associated microorganisms. In particular, tagatose inhibited mycelial growth with severe mitochondrial alterations and stress-related gene modulation in *P. infestans*, but not in *P. cinnamomi* (Chahed et al., 2020), indicating species-specific responses to this rare sugar. Further studies are therefore required, in order to better understand the metabolic and transcriptional reprogramming responsible for the differential inhibitory effects of tagatose on plant-associated microorganisms.

Members of the *Phytophthora* genus are widespread causal agents of destructive diseases for different plant species (Kamoun, 2000). In particular, *P. infestans* and *P. cinnamomi* are two of the most important phytopathogenic oomycetes (Kamoun et al., 2015) and are responsible for severe economic losses in agricultural, horticultural and

forest production (Fry et al., 2015; Hardham, 2005; Kamoun et al., 2015). The control of phytopathogenic oomycetes requires the use of chemical fungicides (Judelson& Blanco, 2005) with potential negative effects on the environment and human health (Fantke, Friedrich, & Jolliet, 2012). Tagatose could represent a valid alternative to chemical fungicides, thanks to its inhibitory properties against *P. infestans* (Chahed et al., 2020) and the absence of negative impacts on human health (Ohara et al., 2008), but further studies are needed to clarify its impacts on metabolic and transcriptional regulations of *Phytophthora* spp. For example, the fungicide ethylicin severely affected amino acid and sugar metabolic processes in *P. infestans* (Zhang et al., 2020). Likewise, changes in the sugar, amino acid and organic acid content were observed in *P. infestans* isolates resistant to the fungicide metalxyl, indicating that fungicide resistance pathways are linked to the modulation of fatty acid biosynthesis and glycerophospholipid metabolism (Maridueña-Zavala, Freire-Peñaherrera, Cevallos- Cevallos, & Peralta, 2017). Transcriptional reprogramming in *Phytophthora* spp. exposed to biofungicides was also found and the natural product melatonin inhibited *P. infestans* growth by the downregulation of genes related to amino acid and sugar metabolism (Zhang et al., 2017). Similarly, the growth inhibition of *P. infestans* caused by *Lysobacter capsici* AZ78 (Tomada et al., 2017) and that of *P. sojae* caused by *Bacillus amyloliquefaciens* JDF3 and *B. subtilis* RSS (Liu et al., 2019) was associated with the downregulation of genes related to protein and sugar metabolism, indicating that complex metabolic responses are activated in *Phytophthora* spp. in response to biological or chemical growth inhibitors. The aim of this study was to elucidate the metabolic changes and transcriptional reprogramming activated by *Phytophthora* spp. in response to tagatose incubation, in order to clarify the differential inhibitory mechanisms of tagatose and the molecular determinants of the species-specific reaction to this rare sugar.

2 | RESULTS

1 | Tagatose affects the mycelial growth, sugar content and amino acid content in *Phytophthora infestans*, but not in *P. cinnamomi*

Tagatose inhibited the growth of *P. infestans* and not that of *P. cinnamomi* (Table 1) at four and ten days after incubation (DAI) on pea agar medium (PAM).

Table 1. Effect of tagatose on *Phytophthora* spp. growth. *Phytophthora infestans* and *P. cinnamomi* growth (mm) was assessed four and ten days after incubation (DAI) on pea agar medium in the absence (control) and presence of tagatose. The two-way analysis of variance (two-way ANOVA) showed no significant differences between the two experimental repetitions ($P > 0.05$, ten replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of 20 replicates from the two experiments are reported for each treatment. Significant differences between tagatose-incubated samples and control samples are marked with an asterisk for each *Phytophthora* spp. and time point, according to the Student's t-test ($P \leq 0.05$).

Species	**Mycelial growth (mm)**					
	4 DAI			10 DAI		
	Control	Tagatose	Significance	Control	Tagatose	Significance
Phytophthora infestans	8.80 ± 0.44	1.39 ± 0.39	*	19.14 ± 0.69	5.02 ± 1.34	*
Phytophthora cinnamomi	32.40 ± 1.28	30.98 ± 1.91		38.5 ± 0.00	38.5 ± 0.00	

In order to investigate impacts of tagatose on *Phytophthora* spp. metabolism, sugar content and amino acid content of *P. infestans* and *P. cinnamomi* mycelium were analysed at 4 and at 10 DAI on PAM in the absence (control) and presence of tagatose (tagatose-incubated) by ion chromatography and high performance liquid chromatography (HPLC), respectively (Figures 1 and S1).

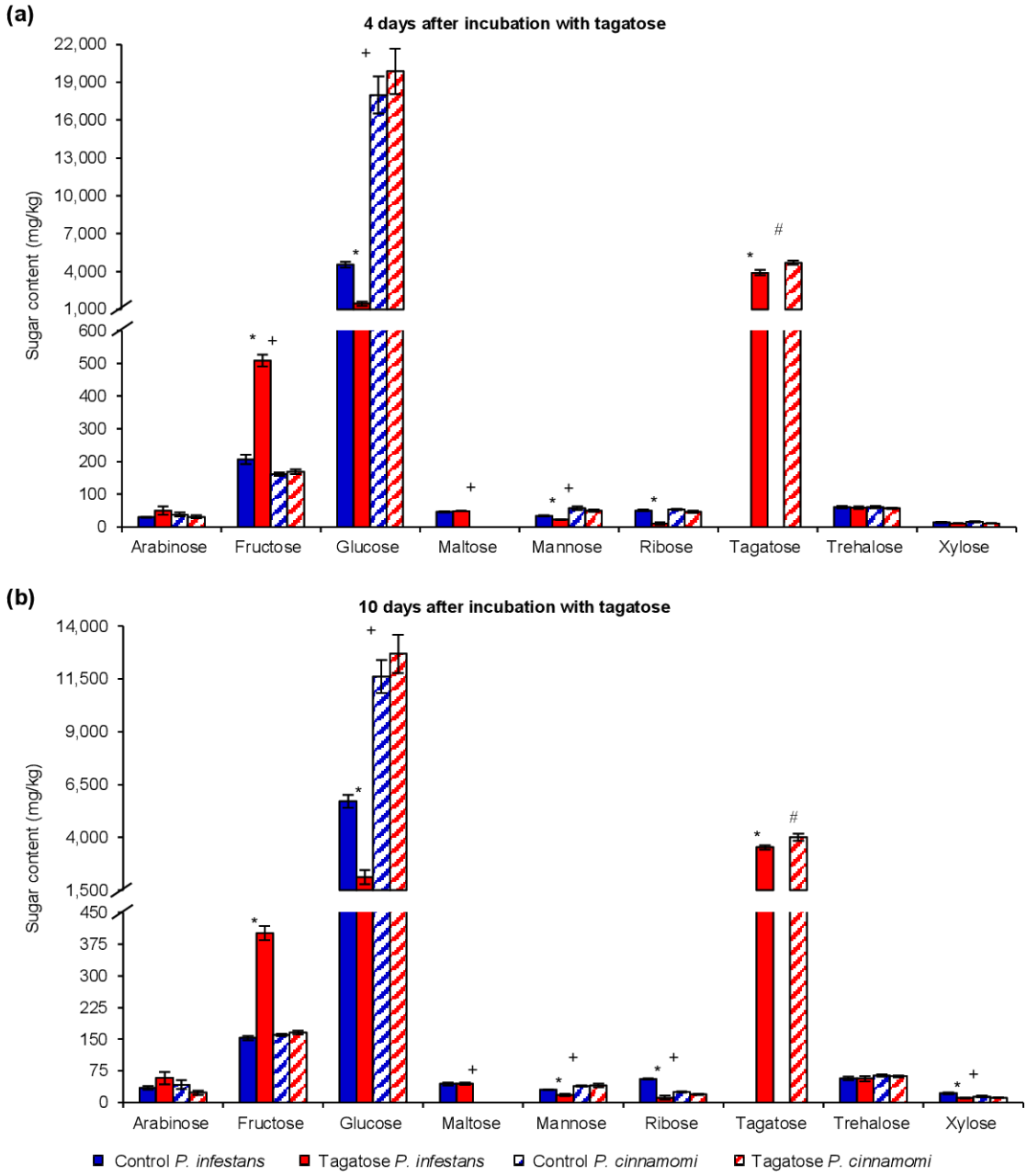


Figure 1. Effect of tagatose on *Phytophthora* spp. sugar content. *Phytophthora infestans* (solid bars) and *P. cinnamomi* (stripped bars) sugar content was quantified at four (a) and ten days (b) after incubation (DAI) on pea agar medium in the absence (control, blue) and presence of tagatose (red). The two-way analysis of variance (two-way ANOVA) showed no significant differences between the two experimental repetitions ($P > 0.05$, three replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of six replicates from the two experiments are presented for each treatment. For each sugar and time point, significant differences between tagatose-incubated samples and control samples are marked with an asterisk (*) for *P. infestans* and a hashtag (#) for *P. cinnamomi*, according to the Student's t-test ($P \leq$

0.05). Significant differences between *P. infestans* control and *P. cinnamomi* control for each sugar are marked with a plus sign (+), according to the Student's t-test ($P \leq 0.05$). Isomaltose, lactose, melibiose and rhamnose were not detected and omitted from the chart.

In *P. infestans*, tagatose incubation decreased the glucose, mannose and ribose content at 4 and at 10 DAI and the xylose content at 10 DAI compared to the respective control samples (Figure 1a,b). Conversely, the fructose content of *P. infestans* was higher in tagatose-incubated samples compared to control samples at 4 and at 10 DAI. In *P. cinnamomi*, the content of each sugar was comparable in tagatose-incubated samples and control samples at 4 and at 10 DAI (Figure 1a,b). As expected, tagatose was found only in tagatose-incubated samples of *P. infestans* and *P. cinnamomi* at both time points, but not in the respective control samples. The sugar content in control samples differed between *P. infestans* and *P. cinnamomi* in terms of glucose, maltose and mannose content at 4 and at 10 DAI, fructose content at 4 DAI, ribose and xylose content at 10 DAI.

The amino acid content was affected by tagatose in *P. infestans* at 4 and at 10 DAI, but only slightly in *P. cinnamomi* at 4 DAI (Figure S1a,b). In *P. infestans*, tagatose incubation decreased the content of 14 amino acids at 4 and at 10 DAI (aminobutyric acid, ethanolamine, glycine, aspartic acid, arginine, asparagine, phenylalanine, glutamine, isoleucine, leucine, ornithine, serine, tryptophan and methionine), the content of two amino acids at 4 DAI (alanine and histidine) and the content of two amino acids at 10 DAI (lysine and tyrosine) compared to the respective control samples. Conversely, the glutamic acid content at 4 and at 10 DAI, or the histidine content at 10 DAI, was higher in tagatose-incubated samples compared to control *P. infestans* samples. In *P. cinnamomi*, the amino acid content was comparable in tagatose-incubated samples and control samples at both time points, except for the lower glutamic acid and glutamine content found in tagatose-incubated samples compared to control samples at 4 DAI.

2 | RNA-Seq analysis reveals species-specific response of *Phytophthora* spp. genes to tagatose incubation

To clarify *Phytophthora* spp. transcriptional regulations in response to tagatose incubation, RNA-Seq analysis was carried out on control and tagatose-incubated samples of *P. infestans* and *P. cinnamomi* at 4 and at 10 DAI. Sequences of the 32 samples [two *Phytophthora* spp. (*P. infestans* and *P. cinnamomi*),two incubation conditions (tagatose-incubated and control), two time points (4 and 10 DAI) and four replicates] were obtained (BioProject number PRJNA622764) and the total number of read pairs that mapped uniquely to the *P. infestans* and *P. cinnamomi* genome ranged from 7,862,604 to 12,711,308 and from 4,197,875 to 9,047,846 for each replicate, respectively (Table S1). The expression level of *P. infestans* and *P. cinnamomi* genes was assessed (Tables S2 and S3) and global effects of tagatose incubation on the *Phytophthora* spp. transcriptome were visualised by principal component analysis (PCA; Figure S2). In particular, PCA discriminated *P. infestans* samples according to the incubation condition in the first principal component (first PC, 81.6%) and according to the time point in the second PC (5.22%; Figure S2a). In the case of *P. cinnamomi*, PCA classified samples according to the time point in the first PC (54.15%) and according to the incubation condition in the second PC (7.73%; Figure S2b). Moreover, the PCA on the putative orthologous genes of *P. infestans* and *P. cinnamomi* (8908 orthologous genes, in total) discriminated samples according to the species in the first PC (72.48%) and highlighted changes between tagatose-incubated samples and control samples of *P. infestans* on the second PC (Figure S2c).

Tagatose incubation resulted in the modulation of 3,901 and 512 differentially expressed genes (DEGs) in *P. infestans* and *P. cinnamomi* respectively, imposing a *P*-value lower than 0.01 and a minimum Log_2 fold change (FC) of one in the pairwise

comparisons between tagatose-incubated samples and control samples at 4 or 10 DAI (Tables S4 and S5). A large fraction (75.9%) of *P. infestans* DEGs and almost half (50.5%) of *P. cinnamomi* DEGs were downregulated by tagatose (Figure S3a,b). DEGs were grouped in upregulated or downregulated genes at both time points (4&10 DAI cluster) and exclusively at 4 DAI (4 DAI cluster) or at 10 DAI (10 DAI cluster) for each *Phytophthora* spp. (Figures S3 and S4). Moreover, 2,172 orthologous DEGs were identified (*P*-value lower than 0.01 and minimum Log_2 FC of one) and they were grouped in 16 clusters (defined by a four-letter code) based on the upregulation (U) or downregulation (D) in tagatose-incubated samples compared to control samples in the two *Phytophthora* spp. and time points, such as modulation in *P. infestans* at 4 DAI (first letter); *P. infestans* at 10 DAI (second letter); *P. cinnamomi* at 4 DAI (third letter); *P. cinnamomi* at 10 DAI (fourth letter; e.g. UUDD cluster includes genes upregulated in *P. infestans* at 4 DAI and 10 DAI and downregulated in *P. cinnamomi* at 4 DAI and 10 DAI; Table S6).

The RNA-Seq results were validated by quantitative real-time PCR (qPCR) analysis of 14 *Phytophthora* spp. genes (Table S7) that were selected according to their expression profiles (six *P. infestans*, six *P. cinnamomi* and two orthologous genes belonging to different clusters) and functional categories (e.g. sugar metabolism, oxidative stress and transport). A close correlation (Pearson r = 0.90) between RNA-Seq and qPCR expression data was observed (Figure S5) and expression profiles agreed completely for 12 genes and differed slightly for two genes (Table S7).

3 | Tagatose incubation causes a severe transcriptional response in *Phytophthora infestans*

A high number of *P. infestans* genes was modulated at 4&10 DAI (2,688 DEGs: 427 upregulated and 2,247 downregulated; Figures S3a and S4a; Table S4). The functional

annotation revealed that upregulated genes of the 4&10 DAI cluster were mainly involved in primary metabolism (e.g. one epoxide hydrolase and two aldehyde dehydrogenases), protein metabolism, transport and energy metabolism (e.g. a fructose-bisphosphate aldolase, three glyceraldehyde-3-phosphate dehydrogenases and two phosphoglycerate mutases; Figure 2a and Table S4).

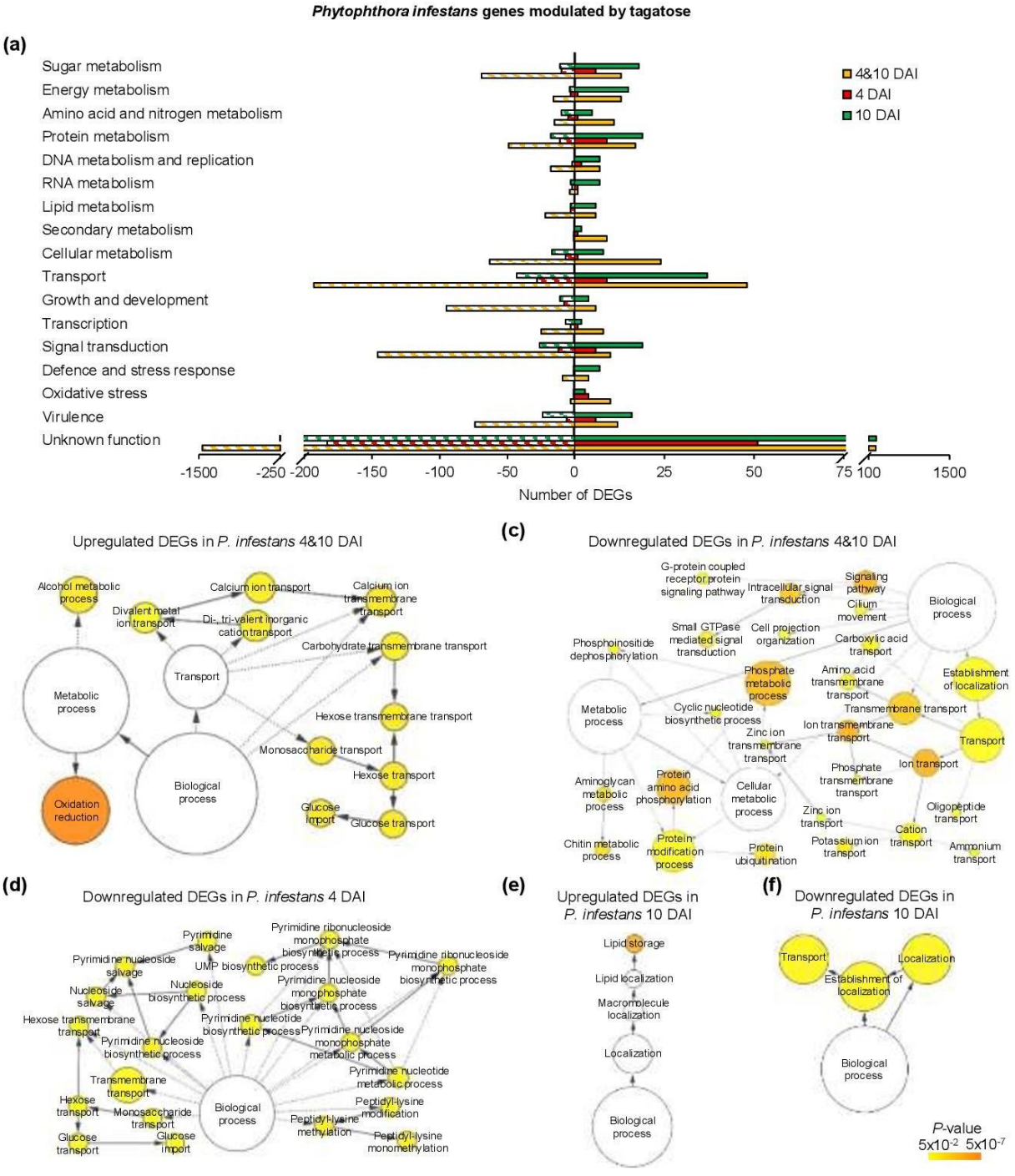


Figure 2. Functional annotation of genes modulated by tagatose in *Phytophthora infestans*. Differentially expressed genes (DEGs) were identified in *P. infestans* at four and ten days after incubation (DAI) on pea agar medium in the presence of tagatose compared to the respective control samples grown in the absence of tagatose. Functional categories (a) were assigned based on the protein description of upregulated (solid bars) or downregulated (stripped bars) genes at both time points (4&10 DAI; orange) and exclusively at 4 DAI (red) or at 10 DAI (green; Table S4). Biological networks of significantly enriched ($P \leq 0.05$) Gene Ontology (GO) terms of *P. infestans* genes upregulated at 4&10 DAI (b), downregulated at 4&10 DAI (c), downregulated at 4 DAI (d), upregulated at 10 DAI (e) and downregulated at 10 DAI (f) were identified using the BiNGO tool and visualised with Cytoscape software. The colour scale legend indicates the level of significance for enriched GO terms and white nodes indicate not significantly overrepresented categories. Dotted lines indicate connection between biological process categories in the GO chart, where ancestor and child are omitted for simplicity. No significant GO enrichment was found for *P. infestans* genes upregulated at 4 DAI.

In particular, the gene ontology (GO) biological process enrichment analysis revealed the overrepresentation of oxidation reduction category (e.g. four glutathione S-transferases and two quinone oxidoreductases) and transport-related processes [e.g. nine ATP-binding cassette (ABC) proteins and seven P-type ATPases (P-ATPase)] in the cluster of upregulated genes at 4&10 DAI (Figure 2b and Table S4). *Phytophthora infestans* genes downregulated by tagatose at 4&10 DAI were mainly implicated in transport (e.g. one glucose transporter, three mitochondrial carriers and three amino acid transporters), signal transduction (e.g. three protein phosphatases and three cyclin-dependent kinases), growth and development (e.g. five myosin-like proteins, five dynein light chains and 21 kinesin-like proteins), sugar metabolism (e.g. an alpha-trehalose-phosphate synthase, a glucokinase, two lysosomal ß-glucosidases, a ß-glucosidase, an endo-1,4-ß-xylanase and 18 glycoside hydrolases) and virulence (Figure 2a and Table S4). Thus, the GO categories related to signalling and transport processes were overrepresented in the cluster of downregulated genes at 4&10 DAI (Figure 2c).

Phytophthora infestans genes modulated at 4 DAI (4 DAI cluster) and at 10 DAI (10 DAI cluster) included 385 DEGs (99 upregulated and 286 downregulated) and 842 DEGs (411 upregulated and 431 downregulated), respectively (Figure S4 and Table S4). Downregulated genes at 4 DAI were mainly involved in transport (e.g. two glucose transporters and two sugar transporters), signal transduction (e.g.two protein kinases) and protein metabolism (e.g. two serine protease inhibitors and three serine proteases; Figure 2a and Table S4). Moreover, the GO categories related to transport (e.g. glucose transport) and pyrimidine metabolism were overrepresented in the cluster of downregulated genes at 4 DAI (Figure 2d and Table S4). Upregulated genes of the 10 DAI cluster were mainly related to protein metabolism (Figure 2a) with the overrepresentation of the lipid storage GO category (Figure 2e and TableS4). Moreover, downregulated genes at 10 DAI were mainly involved in transport (e.g. ten ABC proteins and three mitochondrial carriers), virulence (six crinkler family proteins, one elicitin-like protein and one cutinase) and signal transduction (Figure 2a,f and Table S4). However, a large fraction of genes with an unknown function was modulated in the 4&10 DAI (228 upregulated and 1,445 downregulated), 4 DAI (51 upregulated and 183 downregulated) and 10 DAI (236 upregulated and 252 downregulated) cluster, and indicated that that several yet-to-be identified proteins may have been involved in the *P. infestans* response to tagatose.

In summary, *P. infestans* response to tagatose incubation involved the upregulation of energy metabolism and oxidative stress response. However, the *P. infestans* transcriptional reprogramming was characterised by the downregulation of genes of transport-, sugar metabolism-, signal transduction- and growth-related processes (Figure 3a), in agreement with the inhibition of mycelial growth and alteration of sugar and amino acid content.

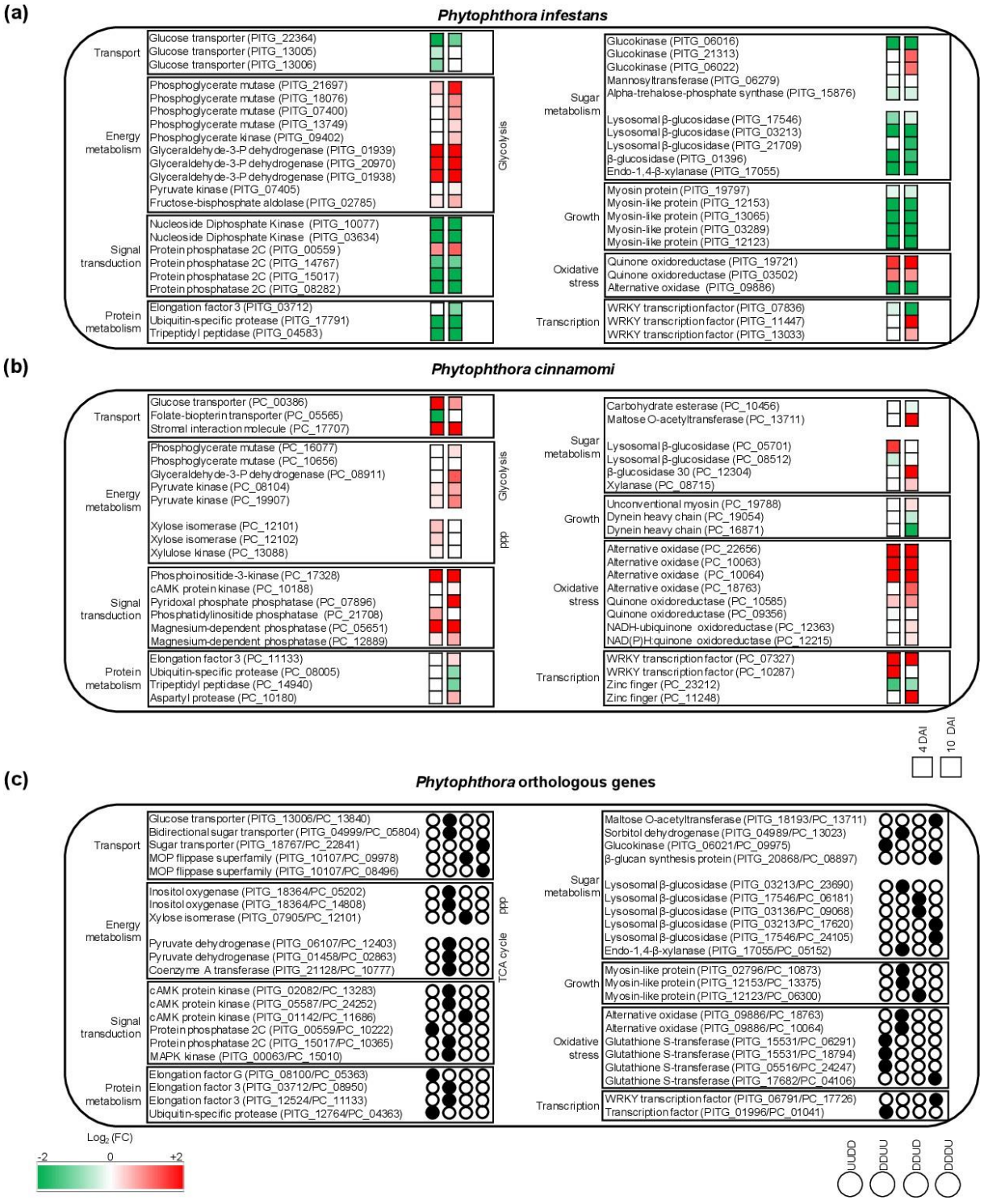


Figure 3. Overview of the main processes modulated by tagatose in *Phytophthor*a spp. Main cellular pathways affected by tagatose were identified for *Phytophthora infestans* (a), *P. cinnamomi* (b) and *Phytophthora* spp. orthologous (c) differentially expressed genes (DEGs) at four and ten days after incubation (DAI) on pea agar medium. For each *P. infestans* and *P. cinnamomi* gene, two squares represent the Log_2-transformed fold change values of tagatose-incubated samples calculated as compared to control samples at 4 (left square) and 10 (right

square) DAI, according to the colour scale reported. Orthologous DEGs were grouped in 16 clusters (defined by a four-letter code, black circles) based on their upregulation (U) or downregulation (D) in tagatose-incubated samples [*P. infestans* at 4 DAI (first letter); *P. infestans* at 10 DAI (second letter); *P. cinnamomi* at 4 DAI (third letter); *P. cinnamomi* at 10 DAI (fourth letter)], such as genes upregulated in *P. infestans* and downregulated in *P. cinnamomi* at 4 DAI and at 10 DAI (UUDD), genes downregulated in *P. infestans* at 4 DAI and at 10 DAI and upregulated in *P. cinnamomi* at 4 DAI and at 10 DAI (DDUU), at 4 DAI (DDUD) or at 10 DAI (DDDU).

4 | Tagatose incubation causes an efficient transcriptional response in *Phytophthora cinnamomi*

The three clusters of *P. cinnamomi* DEGs included 41 genes modulated at 4&10 DAI (36 upregulatedand 5 downregulated), 122 genes modulated at 4 DAI (71 upregulated and 51 downregulated) and 349 genes modulated at 10 DAI (146 upregulated and 203 downregulated; Figures S3b and S4b; Table S5).Although a large fraction of genes with an unknown function was found for *P. cinnamomi* DEGs (17, 78 and 228 in the 4&10 DAI, 4 DAI and 10 DAI cluster, respectively), functional annotations revealed the upregulation of genes involved in signal transduction (e.g. one phosphoinositide-3-kinase and two magnesium-dependent phosphatases), oxidative stress (e.g. three alternative oxidases and one quinone oxidoreductase), energy metabolism (e.g. two pyruvate kinases and one glycerol-3-phosphate dehydrogenase) and transport (e.g. one stromal interaction molecule, one glucose transporter and one voltage-gated potassium channel) in the 4&10 DAI cluster (Figure 4a and Table S5).

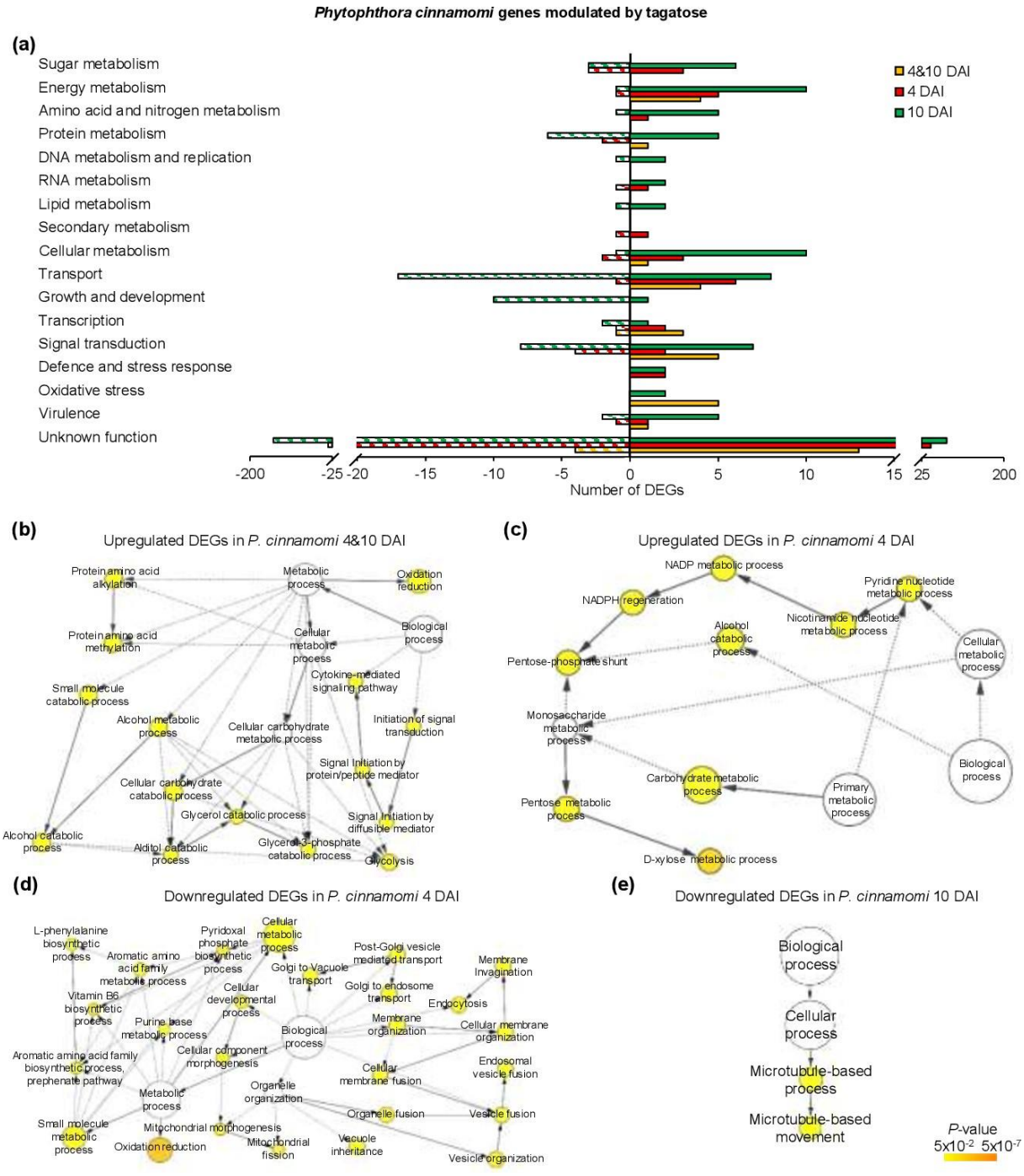


Figure 4. Functional annotation of genes modulated by tagatose in *Phytophthora cinnamomi*. Differentially expressed genes (DEGs) were identified in *P. cinnamomi* at four and ten days after incubation (DAI) on pea agar medium in the presence of tagatose compared to the respective control samples grown in the absence of tagatose. Functional categories (a) were assigned based on the protein description of upregulated (solid bars) or downregulated (stripped bars) genes at both time points (4&10DAI; orange) and exclusively at 4 DAI (red) or at 10 DAI (green; Table S5). Biological networks of significantly enriched ($P \leq 0.05$) Gene Ontology (GO)

terms of *P. cinnamomi* genes upregulated at 4&10 DAI (b), upregulated at 4 DAI (c), downregulated at 4 DAI (d) and downregulated at 10 DAI (e) were identified using the BiNGO tool and visualised with Cytoscape software. The colour scale legend indicates the level of significance for enriched GO terms and white nodes indicate not significantly overrepresented categories. Dotted lines indicate connection between biological process categories in the GO chart, where ancestor and child are omitted for simplicity. No significant GO enrichment was found for *P. cinnamomi* genes downregulated at 4&10 DAI and upregulated at 10 DAI.

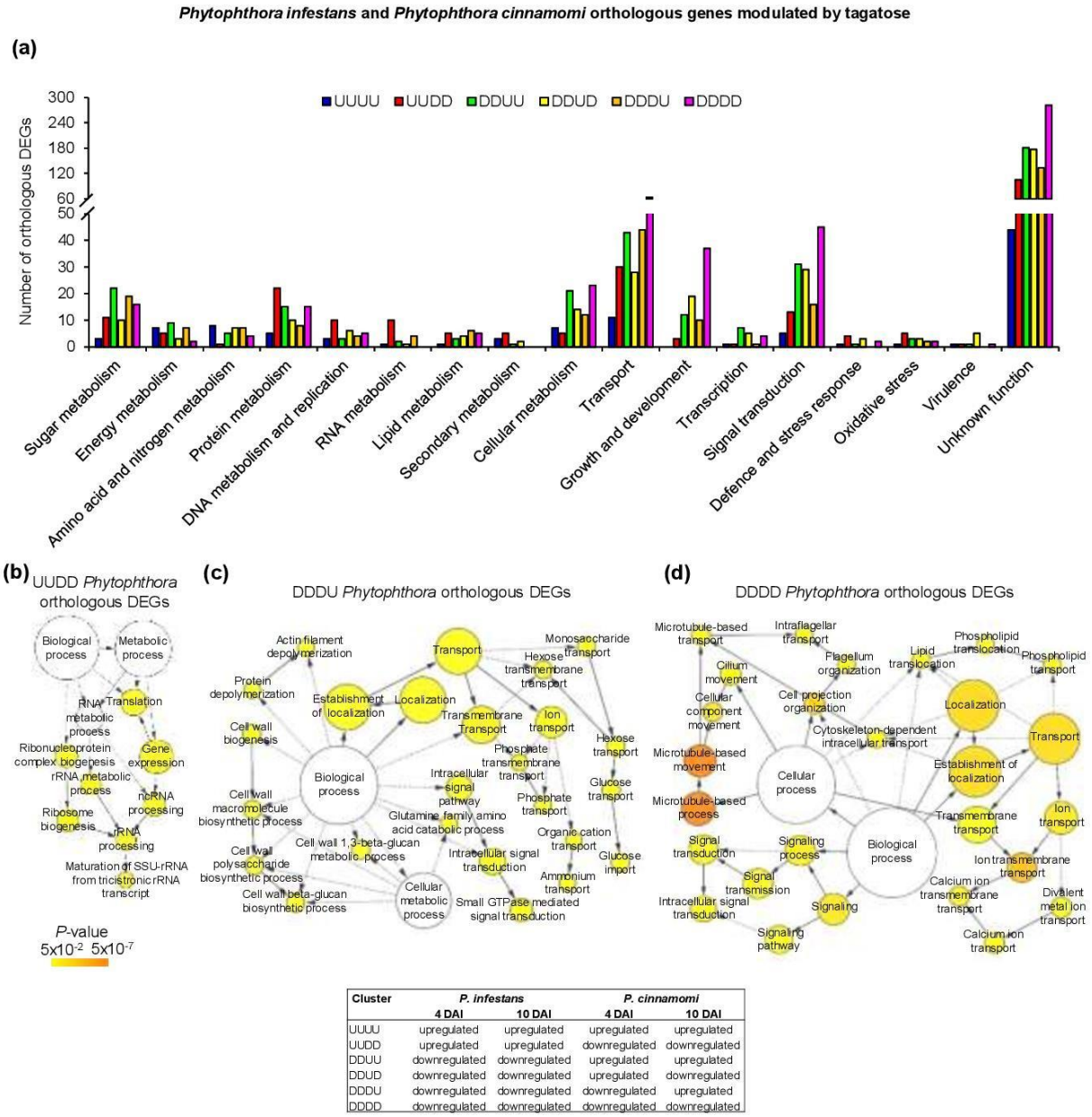
As a consequence, the GO categories related to energy metabolism (e.g. glycerol 3-phosphate catabolic process and glycolosis), oxidation reduction and signalling were overrepresented in the cluster of upregulated genes at 4&10 DAI (Figure 4b and Table S5).

Genes upregulated by tagatose at 4 DAI were mainly involved in transport (e.g. two aquaporins, one MtN3-like protein and one carboxylic acid transporter) and energy metabolism (two xylose isomerases, one xylulose kinase and one succinate dehydrogenase; Figure 4a and Table S5) with the overrepresentation of energy-related GO categories (e.g. NADPH regeneration, pentose metabolic process and carbohydrate metabolic process; Figure 4c and Table S5). On the other hand, downregulated genes at 4 DAI were mainly involved in signal transduction (e.g. two protein kinase and one protein phosphatase) with the overrepresentation of oxidative reduction and vesicle-mediated transport (Figure 4a,d and Table S5). Upregulated genes of the 10 DAI cluster were mainly related to energy metabolism (e.g. two phosphoglycerate mutases, one glyceraldehyde-3-phosphate dehydrogenase and one pyruvate dehydrogenase; Figure 4a and Table S5). Moreover, downregulated genes at 10 DAI were mainly involved in transport, growth and development (Figure 4a), as well as the GO categories of microtubule-based processes (Figure 4e and Table S5). Thus, *P. cinnamomi* response to tagatose was characterised by the upregulation of genes implicated in transport-, energy metabolism- and oxidative stress-related processes (Figure 3b), possibly to adapt the cellular metabolism and

minimise the alteration of the sugarcontent and mycelial growth.

5 | Tagatose differentially modulates orthologous genes in *Phytophthora infestans* and *P. cinnamomi*

Although a large fraction of genes with an unknown function was found for the orthologous DEGs, functional annotations revealed that orthologous genes upregulated in both species and time points (UUUU cluster) were mainly implicated in transport (e.g. one mitochondrial carrier), amino acid and nitrogen metabolism (e.g. one glutamine amidotransferase, three aspartate aminotransferases and one histidinol-phosphate aminotransferase; Figure 5a and Table S6).



Cluster	*P. infestans*		*P. cinnamomi*	
	4 DAI	10 DAI	4 DAI	10 DAI
UUUU	upregulated	upregulated	upregulated	upregulated
UUDD	upregulated	upregulated	downregulated	downregulated
DDUU	downregulated	downregulated	upregulated	upregulated
DDUD	downregulated	downregulated	upregulated	downregulated
DDDU	downregulated	downregulated	downregulated	upregulated
DDDD	downregulated	downregulated	downregulated	downregulated

Figure 5. Functional annotation of *Phytophthora infestans and P. cinnamomi* orthologous genes modulated by tagatose. Orthologous genes of *P. infestans* and *P. cinnamomi* differentially expressed (orthologous DEGs) were identified at four and ten days after incubation (DAI) on pea agar medium in the presence of tagatose compared to the respective control samples grown in the absence of tagatose. Orthologous DEGs were grouped in 16 clusters (defined by a four-letter code) based on their upregulation (U) or downregulation (D) in tagatose-incubated samples (Table S6): *P. infestans* at 4 DAI (first letter); *P.infestans* at 10 DAI (second letter); *P. cinnamomi* at 4 DAI (third letter); *P. cinnamomi* at 10 DAI (fourth letter); such as the cluster of genes upregulated in *P. infestans* and downregulated in *P. cinnamomi* at 4 DAI and 10 DAI (UUDD). Functional categories of the clusters UUUU (blue), UUDD (red), DDUU (green), DDUD (yellow),

DDDU (orange) and DDDD (pink) were assigned based on the protein description (a). Biological networks of significantly enriched ($P \leq 0.05$) Gene Ontology (GO) terms of UUDD (b), DDDU (c) and DDDD (d) cluster of orthologous DEGs were identified using the BiNGO tool and visualised with Cytoscape software. The colour scale legend indicates the level of significance for enriched GO terms and white nodes indicate not significantly overrepresented categories. Dotted lines indicate connection between biological process categories in the GO chart, where ancestor and child are omitted for simplicity. No significant GO enrichment was found for the UUUU, DDUU and DDUD clusters.

Orthologous genes upregulated in *P. infestans* and downregulated in *P. cinnamomi* (UUDD cluster) were mainly related to transport (e.g. six ABC proteins and one voltage-gated ion channel) and protein metabolism (e.g. seven ribosomal genes, one ubiquitin-specific protease and one elongation factor; Figure 5a and Table S6) with the overrepresentation of translation and rRNA metabolic processes (Figure 5b). Moreover, orthologous genes downregulated in *P. infestans* and upregulated in *P. cinnamomi* (DDUU cluster) were mainly associated with sugar metabolism (e.g. seven glycoside hydrolases, one UDP-sugar pyrophospharylase, one sorbitol dehydrogenase, one endo-1,4-ß-xylanase and one lysosomalß-glucosidase), transport (e.g. one glucose transporter, one bidirectional sugar transporter and one glycoside-cation symporter) and signal transduction (Figure 5a and Table S6).

Orthologous genes of the DDUD cluster were mainly linked to transport [e.g. one multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase and two choline transporters), signal transduction, growth and development (e.g. four kinesin-like proteins, one dynein heavy chain and one myosin-like gene; Figure 5a and Table S6). The DDDU cluster consisted of 273 orthologous DEGs that were mainly involved in transport (e.g. two phosphate transporters, a sugar transporter and a MOP flippase) and sugar metabolism (e.g. two lysosomal ß-glucosidases, a maltose O-acetyltransferase and a ß-glucan synthesis protein; Figure 5a and Table S6) with the enrichment of monosaccharide transport and

growth-related processes (e.g. actin filament depolymerization; Figure 5c and Table S6). Orthologous genes downregulated in both species (DDDD cluster) were mainly related to transport, signal transduction, growth and development (Figure 5a and Table S6) with the enrichment of the GO categories of transport, signalling and growth (e.g. microtubule-based process; Figure 5d and Table S6). In summary, differential transcriptional regulation of *Phytophthora* spp. orthologous genes was found inpresence of tagatose, including the activation of protein metabolism in *P. infestans* (UUDD cluster) andthe activation of sugar metabolism, transport, signal transduction and growth-related processes in *P. cinnamomi* (DDUU, DDUD and DDDU cluster; Figure 3c).

3 | DISCUSSION

Tagatose showed nutritional or anti-nutritional properties for specific microbial taxa (Bautista et al., 2000; Perazzolli et al., 2020) and caused differential growth inhibition in some species belonging to The same genus, such as *Phytophthora* (Chahed et al., 2020). In particular, the minimum tagatose dosage showing a differential effects on *Phytophthora* spp. growth was previously identified (5 g/L tagatose; Chahed et al., 2020) and it was used to assess metabolic and transcriptional changes. Differential response to tagatose incubation was found in *P. infestans* and *P. cinnamomi* with species-specific metabolic and transcriptional changes at the time points (4 and 10 DAI) that previously showed cellular ultrastructure changes in *P. infestans*, but not in *P. cinnamomi* (Chahed et al., 2020). In particular, sugar content and amino acid content were affected by tagatose in *P. infestans*, but not in *P. cinnamomi*. Moreover, a high number of genes was modulated by tagatose in *P. infestans* (3,915 DEGs) compared to *P. cinnamomi* (512 DEGs), suggesting that tagatose incubation caused a severe transcriptional reprogramming in *P. infestans*, rather than in *P. cinnamomi*. In addition, *P. infestans* DEGs were mainly repressed (75.70%),

unlike *P. cinnamomi* (50.58%), suggesting global downregulation of functional processes associated with the growth inhibition caused by tagatose in *P. infestans*. Although different growth rates, developmental stages and/or nutrient availability of *Phytophthora* spp. can possibly affect the response to tagatose, metabolic and transcriptional changes of each tagatose-incubated sample were assessed in comparison to the respective control sample, in order to highlight tagatose effects for each time point and each species.

1 | Main cellular processes affected by tagatose incubation in *Phytophthora infestans*

Tagatose incubation impaired *P. infestans* growth and caused severe impacts on sugar content and amino acid content with the downregulation of genes related to transport, sugar metabolism and growth-related process. In particular, tagatose incubation increased the content of fructose and decreased the content of glucose, mannose and ribose in *P. infestans*, but not in *P. cinnamomi*. Tagatose is the C-4 epimer of fructose and it may inhibit fructose-metabolising enzymes in *P. infestans*, as previously reported for the fructokinase in mammalian (Lu, Levin, & Donner, 2008) and in *Hyaloperonospora arabidopsidis* (Mochizuki et al., 2020), with the consequent increase of the fructose content. On the other hand, the decrease in glucose content could be ascribed to the tagatose-depended inhibition of *P. infestans* ß- glucosidase enzymes (Corneo et al., 2020), which are involved in polysaccharide and disaccharide hydrolysis (Saha, Freer, & Bothast, 1994). As corroboration, genes encoding ß-glucosidase enzymes were mainly down- and up-regulated by tagatose in *P. infestans* and *P. cinnamomi*, respectively. In agreement with these findings, previous transcriptomic studies showed that the growth inhibition of *Phytophthora* spp., caused by biological products (melatonin), biocontrol bacteria (*L. capsici* AZ78) and fungicides (dimethomorph and metalaxyl), was associated with the downregulation of genes related to sugar metabolism (Hao et al., 2019; Tomada et al., 2017;

Zhang et al., 2017) and to the decrease in sugar content (Maridueña-Zavala et al., 2017), indicating a strong correlation between sugar metabolism and *P. infestans* growth. Likewise, sugar- and growth-related genes involved in cell wall integrity and biogenesis (e.g. trehalose synthase, glucanosyltrasnferase, glucose 4,6-dehydratases and β-glucan synthase genes) were downregulated by tagatose in *P. infestans*, but not in *P. cinnamomi*. The impact of tagatose on oomycete cell wall was previously reported in *H. arabidopsidis*, where tagatose inhibited the metabolism of mannan (Mochizuki et al., 2020), which is an essential component of oomycete cell wall (Melida, Sandoval-Sierra, Dieguez-Uribeondo, & Bulone, 2013). The alteration of cell wall integrity can negatively affect *P. infestans* pathogenicity (Resjö et al., 2017), and genes related to virulence and infection processes, such as RxLR (Kamoun, 2006), necrosis-inducing protein (NPP1) (Qutob, Kamoun,& Gijzen, 2002) and thrombospondin (Robold & Hardham, 2005) were downregulated in tagatose- incubated *P. infestans*. Although possible alterations on pathogenicity should be validated by artificial inoculations on tomato plants, transcriptional changes indicated severe impacts of tagatose on key functional processes of *P. infestans*.

As a possible consequence of sugar metabolism inhibition, tagatose incubation decreased amino acid content in *P. infestans*, but not in *P. cinnamomi*. Previous studies showed that some fungal species (e.g.*Candida albicans*) can exploit amino acids as a carbon source under sugar-limiting conditions (Ene, Brunke, Brown, & Hube, 2014), suggesting that *P. infestans* could catabolise amino acids in the presence of tagatose. The amino acid catabolism involves cytoplasmic transaminases to form glutamic acid (Judelson, Tani, & Narayan, 2009), which is the only amino acid to increase in tagatose-incubated *P. infestans*. As corroboration, genes involved in glutamic acid biosynthesis (e.g. glutamine amidotransferase and histidinol-phosphate transaminase genes) were upregulated

by tagatose, suggesting that amino acid metabolism was reprogrammed in *P. infestans* possibly to compensate the inhibition of sugar metabolism. Likewise, the growth inhibition of *Phytophthora* spp. caused by biological products (melatonin) and fungicides (e.g. ethylicin and SYP-14288) was previously associated with the inhibitionof amino acid metabolism and protein synthesis (Cai et al., 2019; Zhang et al., 2017; Zhang et al., 2020). For example, arginine content was decreased in tagatose-incubated *P. infestans* and genes involved in arginine biosynthesis (e.g. arginino-succinate synthase) were downregulated. The decrease of amino acidcontent was associated also with the downregulation of amino acid transporters in tagatose-incubated *P.infestans*. Similarly, the downregulation of amino acid transporter genes was previously linked to *P. infestans* growth inhibition caused by *Pseudomonas fluorescens* LBUM223 (Roquigny, Novinscak, Arseneault, Joly, & Filion, 2018), indicating strong correlations between sugar metabolism, amino acid metabolism, amino acid transport and *P. infestans* growth.

2 | Processes with opposite modulation in *Phytophthora infestans* and in *P. cinnamomi* after tagatose incubation

Opposite modulation of genes involved in transport, energy metabolism, growth-related process, and oxidative stress response were found in *P. infestans* and *P. cinnamomi*, indicating species-specific reaction to tagatose incubation. In particular, tagatose incubation led to the down- and up-regulation of genes encoding sugar transporters (e.g. glucose transporters, bidirectional sugar transporters, glycoside-pentoside transporters and multidrug/oligosaccharidyl-lipid/polysaccharide flippases) in *P. infestans* and *P. cinnamomi*, respectively. Clinical studies showed that tagatose may act by attenuating glucose absorption in the human intestine (Donner, Wilber, & Ostrowski, 1999). Likewise, the rare sugar sorbose inhibited glucose transport in *Saccharomyces cerevisiae* leading to

growth retardation (Van Uden, 1967), suggesting that the growth inhibitory effects of rare sugars may be mediated by glucose transport inhibition. Thus, the upregulation of glucose and sugar transporters in *P. cinnamomi* could be an efficient cellular response to mitigate tagatose inhibitory effects on sugar transporters and to maintain sufficient glucose uptake and energy production. Likewise, genes implicated in the tricarboxylic acid (TCA) cycle (e.g. malate synthase, succinate-semialdehyde dehydrogenase, succinate dehydrogenase, pyruvate dehydrogenase and pyruvate dehydrogenase) and mitochondrial respiration (e.g. ubiquinone biosynthesisprotein COQ7, cytochrome b5 and, NADH dehydrogenase), were down- and up-regulated by tagatose incubation in *P. infestans* and *P. cinnamomi*, respectively. In agreement with these findings, tagatose incubation decreased the content of TCA cycle intermediates in *P. infestans* (such as malic acid and succinic acid) (Corneo et al., 2020) and impaired normal respiration and ATP synthesis in *P. infestans*, but not in *P. cinnamomi* (Chahed et al., 2020), indicating species-specific effects of tagatose on some pathways of energy metabolism. As a possible consequence of energy limitation, genes associated with growth and development (e.g. myosin-like protein and actin-like protein) were mainly down- and up- regulated by tagatose incubation in *P. infestans* and *P. cinnamomi*, respectively. Likewise, *Phytophthora capsici* growth inhibition by the fungicide benzothiazole was previously associated with the downregulation of growth-related genes, such as those encoding actin and ankyrin repeat-protein (Mei et al., 2019).

Although genes involved in oxidative stress response were upregulated by tagatose incubation in both *Phytophthora* spp., some genes belonging to this functional category showed species-specific profiles. For example, glutathione S-transferase genes were up- and down-regulated in *P. infestans* and *P. cinnamomi* respectively (UUDD cluster), while alternative oxidase genes showed opposite profile (DDUU cluster). Glutathione S-transferase gene is a marker of oxidative stress and it was induced by biocontrol bacteria

(*L. capsici* AZ78 and *P. fluorescens* LBUM223) in *P. infestans* (Roquigny et al., 2018; Tomada et al., 2017). Thus, expression profiles of glutathione S-transferase genes agreed with the stronger accumulation of reactive oxygen species (ROS) previously found in *P. infestans* compared to *P. cinnamomi* during tagatose incubation (Chahed et al., 2020). Conversely, the upregulation of alternative oxidase genes in *P. cinnamomi* rather than *P. infestans* suggest a key contribution of these enzymes in the ROS detoxification, as previously found for the oxidative stress-handling machinery of *Ustilago maydis* (Juarez et al., 2006). Alternative oxidases are also key enzymes of the alternative respiration pathways in phytopathogenic fungi (Tian, Lee, Woo, & Chun, 2020) and their expression was upregulated by non-fermentable carbon sources (e.g. glycerol and ethanol) in *C. albicans* (Huh & Kang, 2001). Thus, *P. cinnamomi* may activate alternative oxidase pathways, in order to mitigate the oxidative stress and to allow alternative respiration, in agreement with the normal ROS level and oxygen consumption rate previously found during tagatose incubation (Chahed et al., 2020). Moreover, genes related to signal transduction were mainly down- and up-regulated in *P. infestans* (e.g. protein phosphatases 2C and cAMP kinases) and *P. cinnamomi*, respectively. Phosphatase 2C genes were downregulated by oxidative stress in *Nicotiana tabacum* (Vranová, Langebartels, Montagu, Inzé, & Camp, 2000) and signalling genes (e.g. cAMP domain-containing protein) were downregulated by growth inhibition in *P. capsici* (Mei et al., 2019), suggesting the activation of complementary signal transduction pathways in *P. infestans* and *P. cinnamomi* to modulate the response to tagatose incubation.

3 | Main cellular processes affected by tagatose incubation in *Phytophthora cinnamomi*

Mycelial growth, sugar and amino acid content were not affected by tagatose incubation in *P. cinnamomi* and the transcriptional response revealed the upregulation of genes related

to transport, energy metabolism, sugar metabolism and oxidative stress. In particular, the response of *P. cinnamomi* included the upregulation of genes related to pentose metabolism, such as xylose isomerase, xylulose kinase and inositol oxygenase, indicating the activation of alternative sugar catabolism. Previous studies showed that blocking hexose entry into glycolysis upregulated pentose metabolism in *Aspergillus nidulans* (Khosravi et al., 2018) and that xylulose kinase co-expression with arabinan-degrading genes was essential for *Aspergillus niger* growth on xylose and arabinose (VanKuyk, De Groot, Ruijter, De Vries, & Visser, 2001). Likewise, genes encoding sorbitol dehydrogenase and arabinan-degrading enzymes responsible for the release of pentose sugars from polysaccharides side chains (e.g. endo-1,4-beta- xylanase, arabinogalactan endo-beta-1,4-galactanase, arabinan endo-1,5-alpha-L-arabinosidase) were upregulated by tagatose incubation in *P. cinnamomi*. Consequently, the pentose sugars originated by these enzymatic activities can be assimilated into the pentose phosphate pathway, suggesting that *P. cinnamomi* metabolised alternative sugars and activated the pentose metabolism to alleviate metabolic impacts caused by tagatose. In addition, the *P. cinnamomi* response included the upregulation of a mannitol dehydrogenase gene, which was upregulated in fungicide-resistant *Phytophthora* isolates (Childers et al., 2015), and the upregulation of transcription factors (e.g. WRKY, zink finger proteins and MYB-like DNA-binding protein), suggesting the activation of an efficient machinery to modulate the response to tagatose incubation.

4 | Processes commonly affected by tagatose incubation in both *Phytophthora* spp.

Some oxidative stress-related genes (e.g. quinone oxidoreductase) were upregulated in *P. infestans* and in *P. cinnamomi*, indicating that both species incurred an oxidative stress after tagatose incubation. However, the efficient scavenging of reactive oxygen species

found in *P. cinnamomi* (Chahed et al., 2020) was associated with the upregulation of additional genes (such as alternative oxidases discussed above), which were downregulated in *P. infestans*. In both *Phytophthora* spp., some genes involved in the energy metabolism of glycolysis (e.g. phosphoglycerate mutase, glyceraldehyde-3-P dehydrogenaseand pyruvate kinase) were upregulated by tagatose incubation. Tagatose was patented as glycolysis inhibitor in humans (Kim et al., 2014) and showed inhibitory effects on glycolysis-related enzymes in *H. arabidopsidis* (fructokinase and phosphomannose isomerase) (Mochizuki et al., 2020) and *E. coli* (fructose phosphate aldolase) (Stellmacher et al., 2016). As a possible consequence of glycolysis inhibition, *P. infestans* and *P. cinnamomi* upregulated a pyruvate phosphate dikinase gene (cluster UUUU) to generate pyruvate, as a bypass of pyruvate kinase in glycolysis reactions (Judelson et al., 2009). Likewise, genes encoding glycerol-3-phosphate dehydrogenase were upregulated in both *Phytophthora* spp. and they can reinforce glycolysis pathways by providing an extra source of dihydroxyacetone phosphate (Lalle et al., 2015), as an attempted reaction to alleviate metabolic impacts derived from the glycolysis inhibition. The enzymatic inhibition caused by tagatose can be ascribed to the structural similarity with fructose and to the possible interference with the substrate binding by the active site, as in the case of the mammalian fructokinase (Lu et al., 2008) and *S. mutans* glucosyltransferase (Hasibul et al., 2018). This competitive effect was also suggested by the fructose-dependent attenuationof tagatose effects in *P. infestans* (Corneo et al., 2020) and in *S. mutants* (Hasibul et al., 2018), but further enzymatic studies are required in order to better clarify the inhibitory effects of tagatose on *Phytophthora* spp. enzymes. Likewise, metabolic and transcriptomic studies at early time points in presence of different dosages of rare sugars and/or common sugars will be required, in order to investigate the early response of *Phytophthora* spp. to tagatose and to clarify possible dose-dependent

interactions between rare sugar and common sugar metabolism.

4 | CONCLUSION

The differential inhibitory effect of tagatose on *P. infestans* and *P. cinnamomi* was associated with species-specific metabolic and transcriptional changes. In particular, an attempted reaction was activated by *P. infestans*, but it was not sufficient to contrast the negative effects of tagatose incubation on mycelial growth, sugar content and amino acid content. Thus, *P. infestans* transcriptional reprogramming was mainly characterised by the severe downregulation of genes implicated in transport, sugar metabolism, signal transduction and growth-related process. Conversely, *P. cinnamomi* response to tagatose incubation was characterized by the activation of processes related to transport, energy metabolism, sugar metabolism and oxidative stress-related, in order to limit negative impacts on mycelial growth, sugar content and amino acid content. In particular, *P. cinnamomi* was able to implement multiple pathways to modulate the cellular metabolism based on the upregulation of genes related to glucose transport, pentose metabolism, TCA cycle, ROS detoxification, mitochondrial and alternative respiration. These metabolicand transcriptional results represent a major contribution to the characterization of the species-specific mode of action of tagatose on *Phytophthora* spp. and might pave the way for functional genomic and biochemical studies to further characterise enzymatic reactions affected by this rare sugar.

5 | EXPERIMENTAL PROCEDURES

1 | *Phytophthora* spp. growth conditions and tagatose incubation

Phytophthora infestans strain VB3 and *P. cinnamomi* strain CBS 144.22 were stored in glycerol at -80°C in the fungal collection of the Fondazione Edmund Mach and they are

available upon request. *Phytophthora infestans* and *P. cinnamomi* were grown in Petri dishes on PAM (12.5% frozen peas and 1.2% agar in distilled water) at 18°C and 25°C in dark conditions, respectively (Chahed et al., 2020). The stock solution (50 g/L in distilled water) of tagatose (Bi-PA, Londerzeel, Belgium) was filter sterilised and added at the final concentration of 5 g/L on PAM shortly before *Phytophthora* spp. inoculation, as reported by Chahed et al. (2020).

Phytophthora spp. growth was assessed as previously described (Chahed et al., 2020). Briefly, a plug (5 mm diameter and 1 mm height) of a 14 days-old colony was placed at the centre of each dish (90mm diameter) on PAM in the absence (control) or presence of 5 g/L tagatose. The radial growth of *P. infestans* and *P. cinnamomi* was assessed at 4 and at 10 DAI at 18°C and 25°C respectively, calculated as the average of the two perpendicular diameters of the colony, minus the plug diameter and the result divided by two. Ten replicates (dishes) were used for each treatment and the experiment was carried out twice.

For metabolic and transcriptomic analyses, a plug (5 mm diameter and 2 mm height) of a 14 days- old colony was placed on PAM covered with sterile cellophane layers in the absence (control) and presence of 5 g/L tagatose. *Phytophthora infestans* and *P. cinnamomi* samples were collected at 4 and at10 DAI after incubation at 18°C and 25°C, respectively, as previously described (Chahed et al., 2020). Briefly, mycelium samples were collected with a sterile forceps, transferred in a sterile 50 mL-tube, immediately frozen in liquid nitrogen and stored at -80°C. Samples were crushed using a mixer mill disruptor (MM200, Retsch, Haan, Germany) at 25 Hz for 45 seconds with sterile steel jars and beads refrigerated in liquid nitrogen.

2 | Sugar and amino acid quantification

Ground *Phytophthora* spp. mycelium (500 mg) or uninoculated PAM (500 mg; Table S8)

were dissolved in 25 mL of ultrapure water, filtered through a 0.22 µm PTFE membrane (Sartorius, Goettingen, Germany) and used for sugar and amino acid quantification (Chemistry Unit at Fondazione Edmund Mach).

The sugar content (arabinose, fructose, glucose, isomaltose, lactose, maltose, mannose, melibiose, rhamnose, ribose, tagatose, trehalose and xylose) was assessed by ion chromatography (Cataldi, Campa,& De Benedetto, 2000) and it was expressed as quantity of each sugar per unit of mycelium fresh weight (mg/kg), using a calibration curve of each pure sugar (Sigma-Aldrich, Merc, Kenilworth, NJ, USA) dissolved in ultrapure water within a range between 0.4 and 40 µg/mL. Briefly, *Phytophthora* spp. mycelium samples, uninoculated PAM samples and calibration curves of each pure sugar were analysed with an ionic chromatograph ICS 5000 (Dionex-Thermo Scientific, Waltham, MA, USA), equipped withan autosampler, a quaternary gradient pump, a column oven and a pulsed amperometric detector with a gold working electrode and a palladium counter electrode. The separation was obtained by injecting 5 µl of each *Phytophthora* spp. mycelium sample, uninoculated PAM sample or pure sugar onto a CarboPacPA200 3 × 250 mm analytical column (Dionex, Thermo Scientific, Waltham, MA, USA), preceded by a CarboPac PA200 3 × 50 mm guard column (Dionex, Thermo Scientific), with a NaOH gradient (from 1 to 30 mM) at 0.4 mL/min flow rate.

The amino acid content (aminobutyric acid, ethanolamine, glycine, glutamic acid, aspartic acid, alanine, arginine, asparagine, citrulline, phenylalanine, glutamine, isoleucine, histidine, leucine, lysine, ornithine, serine, tyrosine, threonine, valine, tryptophan and methionine) was assessed by HPLC (Hill, Walters, Wilson, & Stuart, 1979) and it was expressed as quantity of each amino acid per unit of mycelium fresh weight (mg/kg), using a calibration curve of each pure amino acid (Sigma-Aldrich, Merc) dissolved in ultrapure water within a range between 0.1 and 50 µg/mL. Briefly, *Phytophthora* spp.

mycelium samples, uninoculated PAM samples and calibration curves of each amino acid were analysed with a HPLC instrument (1100 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a degasser, a quaternary gradient pump, an autosampler, a thermostatted column oven and a Fluorimetric Detector (FLD). The chromatographic separation of amino acids was obtained by injecting 5 µl of each *Phytophthora* spp. mycelium sample, uninoculated PAM sample or pure amino acid onto a Chromolith Performance RP-18e column (100 × 4.6 mm) and a guard RP-18e column (10 × 4.6 mm; Merck, Kenilworth, NJ, USA) kept at 40°C with an eluent flow of 2.0 mL/min. The FLD was set at 336 nm as excitation and at 445 nm as emission wavelength.

For sugar and amino acid quantification, three replicates (each replicate obtained as a pool of ten dishes) were analysed for each *Phytophthora* spp., treatment and time point, and the experiment was carried out twice.

3 | RNA extraction, sequencing and mapping to reference genomes

Total RNA was extracted from ground *Phytophthora* spp. mycelium (100 mg) using the Spectrum Plant total RNA kit (Sigma-Aldrich, Merck) with an on-column DNase treatment using the RNase-Free DNaseSet (Qiagen, Hilden, Germany). Total RNA was quantified using a Qubit (Thermo Fisher Scientific) and RNA quality was checked using a Bioanalyzer 2100 (Agilent Technologies). Four replicates (each replicate obtained as a pool of ten dishes) were analysed for each *Phytophthora* spp., treatment and timepoint. RNA samples were subjected to RNA-Seq library construction, using the TruSeq Stranded Total RNA Library Prep Plant protocol (Illumina, SanDiego, CA, USA) with rRNA depletion with the RiboZero Plant kit (Tomada et al., 2017) according to the manufacturer's instructions. Paired-end reads of 150 bases were obtained using a HiSeq 2500 (Illumina) at the Institute of Applied Genomics (Udine,Italy).

Raw Illumina reads were cleaned and filtered using Trimmomatic programme version 0.36 (Bolger,Lohse, & Usadel, 2014) and low-quality bases with an average Phred quality score lower than 15 in a sliding window of four base were removed. Reads shorter than 36 bases in length were removed from the analysis and quality check of raw reads was performed using Fast QC version 0.11.7. Read pairs of *P. infestans* samples were aligned to the *P. infestans* ASM14294v1 genome (https://protists.ensembl.org/Phytophthora_infestans/Info/Index) using the STAR V2.7 program (Dobinet al., 2013), while those of *P. cinnamomi* samples were aligned to *P. cinnamomi* V1.0 genome (https://mycocosm.jgi.doe.gov/Phyci1/Phyci1.home.html). Counts of unambiguously mapped read pairs were obtained during the alignment with the STAR V2.7 program. Putative orthologous genes of *P. infestans* and *P. cinnamomi* (orthologous genes) were identified by reciprocal best BLAST hit using the BLAST+ 2.7.1 program (Camacho et al., 2009) with a threshold of 70% identity and 50% alignment length on amino acid sequences, in order to better analyse the differential *Phytophthora* spp. response.

4 | Identification and functional annotation of differentially expressed genes

DEGs were identified with the Limma-Voom package (Law, Chen, Shi, & Smyth, 2014) which estimates the mean–variance relationship of log counts, generating a precision weight for each observation that is fed into the Limma empirical Bayes analysis pipeline (Smyth, 2004). A Volcano Plot (Patterson et al., 2006) was generated using the Python programming language and the matplotlib package (Hunter, 2007) and a double cutoff on *P*-value ($P \leq 0.01$) and minimum Log_2 FC of one [Log_2 (FC) ≥ 1 or Log_2 (FC) ≤ -1] was used to select DEGs as previously reported (Shen et al., 2020; Tomada et al., 2017). Four pairwise comparisons were analysed, in order to identify DEGs in each *Phytophthora* spp.:

tagatose-incubated *vs.* control of *P. infestans* at 4 DAI, tagatose-incubated *vs.* control of *P. infestans* at 10 DAI, tagatose- incubated *vs.* control of *P. cinnamomi* at 4 DAI, tagatose-incubated *vs.* control of *P. cinnamomi* at 10 DAI. The distribution of *P. infestans* and *P. cinnamomi* DEGs was summarised using Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) and DEGs were grouped in upregulated genes [$P \leq 0.01$ and $Log_2(FC) \geq 1$] or downregulated genes [$P \leq 0.01$ and $Log_2(FC) \leq -1$] at both time points (4&10 DAI cluster) and exclusively at 4 DAI (4 DAI cluster) or at 10 DAI (10 DAI cluster) for each *Phytophthora* spp. The heat map diagram of Log_2-transformed FC values of *Phytophthora* spp. DEGs was visualised using the Java Treeview tool (Saldanha, 2004).

On the list of *Phytophthora* orthologous genes (8,908 in total), orthologous DEGs were identified imposing a *P*-value lower than 0.01 and minimum Log_2 FC of one using a Volcano Plot with the Python programming language and the matplotlib package (Hunter, 2007) in the pairwise comparisons between tagatose-incubated samples and control samples at 4 DAI and at 10 DAI for each *Phytophthora* spp. Contrasts were defined in order to identify genes that respond differently in *P. infestans* compared to *P.cinnamomi* in the comparison between control and tagatose-incubated samples at 4 and at 10 DAI. Orthologous DEGs were then grouped in 16 clusters (defined by a four-letter code) based on their upregulation (U) or downregulation (D) in tagatose-incubated samples compared to control samples in the two *Phytophthora* spp. at 4 and at 10 DAI (e.g. UUDD cluster includes genes upregulated in *P. infestans* at 4 DAI and 10 DAI and downregulated in *P. cinnamomi* at 4 DAI and 10 DAI).

A PCA was performed using the Phyton programming language and the scikit.learn Phyton package (www.python.org) on Limma-normalized expression values. Gene sequences of *P. infestans* and *P. cinnamomi* were aligned against the UniProtKB database

(downloaded on May 2019 from http://www.uniprot.org) using a BLAST-X search and the three or five best protein hits with an E-value lower than 1×10^{-5} were selected for functional annotation. In particular, DEGs were annotated based on the *Phytophthora* spp. protein description resulted by BLAST-X search and grouped into 17 functional categories according to the previous literature. *Phytophthora* spp. genes were further annotated using ARGOT2 function prediction tool for the Gene Ontology functional annotation (Falda et al., 2012). GO terms significantly overrepresented ($P \leq 0.05$, Benjamin and Hochberg FDR correction) in the DEG lists in comparison to the whole transcriptome of the respective *Phytophthora* spp. were identified using the Biological Networks Gene Ontology (BiNGO) tool (Maere, Heymans, & Kuiper, 2005) and biological networks were visualised with Cytoscape version 3.7.2 (Shannon et al., 2003).

5 | Gene expression analysis by quantitative real-time RT-PCR

Phytophthora spp. genes were selected for qPCR analysis (Table S7). A primer pair compatible for the *P. infestans* and *P. cinnamomi* sequence was designed on conserved coding regions in the case of orthologous genes. The first strand cDNA was synthesised from 1 µg of DNase-treated RNA using Superscript III (Invitrogen, Thermo Fisher Scientific) and the oligo-dT primer. qPCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific) and specific primers using the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany), as previously described (Chahed et al., 2020). Briefly, the PCR conditions were: 50°C for 2 min and 95°C for 2 min as initial steps, followed by 50 cycles of 95 °C for 15 s and 60°C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analysed to verify the specificity of each amplification reaction. Light Cycler 480 SV1.5.0 software (Roche) was used to extract Ct values based on the second derivative calculation and LinReg software

was used to calculate reaction efficiencies (Ruijter et al., 2009). The relative expression level (FC) of each gene was calculated according to the Pfaffl equation (Pfaffl, 2001) for tagatose-incubated samples compared to the respective control samples (calibrator) for each *Phytophthora* spp. and time point. The gene encoding β-tubulin (*tub-b*) was used as constitutive gene for the expression level normalization (Yan & Liou, 2006), because its expression was not significantly affected by the treatments (Table S7). Four replicates (each replicate obtained as a pool of ten dishes) were analysed for each *Phytophthora* spp., treatment and time point.

6 | Statistical analysis

Mycelial growth, sugar and amino acid data were analysed with Statistica 13.1 software (TIBCO Software, Palo Alto, CA, USA). Normal distribution (Kolmogorov–Smirnov test, $P > 0.05$) and variance homogeneity of the data (Levene's tests, $P > 0.05$) were checked and parametric tests were used when both assumptions were respected. Each experimental repetition was analysed singularly and a two-way analysis of variance (two-way ANOVA) was used to demonstrate non-significant differences between the two experiments ($P > 0.05$). Data from the two experimental repetitions were pooled and significant differences were assessed with the Student's t-test ($P \leq 0.05$) or Tukey' test ($P \leq 0.05$) in case of pairwise or multiple comparisons, respectively.

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CONFLICT OF INTEREST

AC and AN were employed by Bi-PA nv. The remaining authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

AC and AN carried out the experiments, sample collection and RNA extraction. VL carried out the RT-qPCR experiments. MM analysed the RNA-Seq data. PEC helped to carry out experiments and sample collection. AC, MP, EAB, GP and IP contributed to data interpretation and manuscript writing. MP conceived the study, designed the experiment and coordinated all research activities. All the authors revised and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

The sequences were deposited at the Sequence Read Archive of the National Center for Biotechnology under the BioProject number PRJNA622764.

SUPPORTING INFORMATION

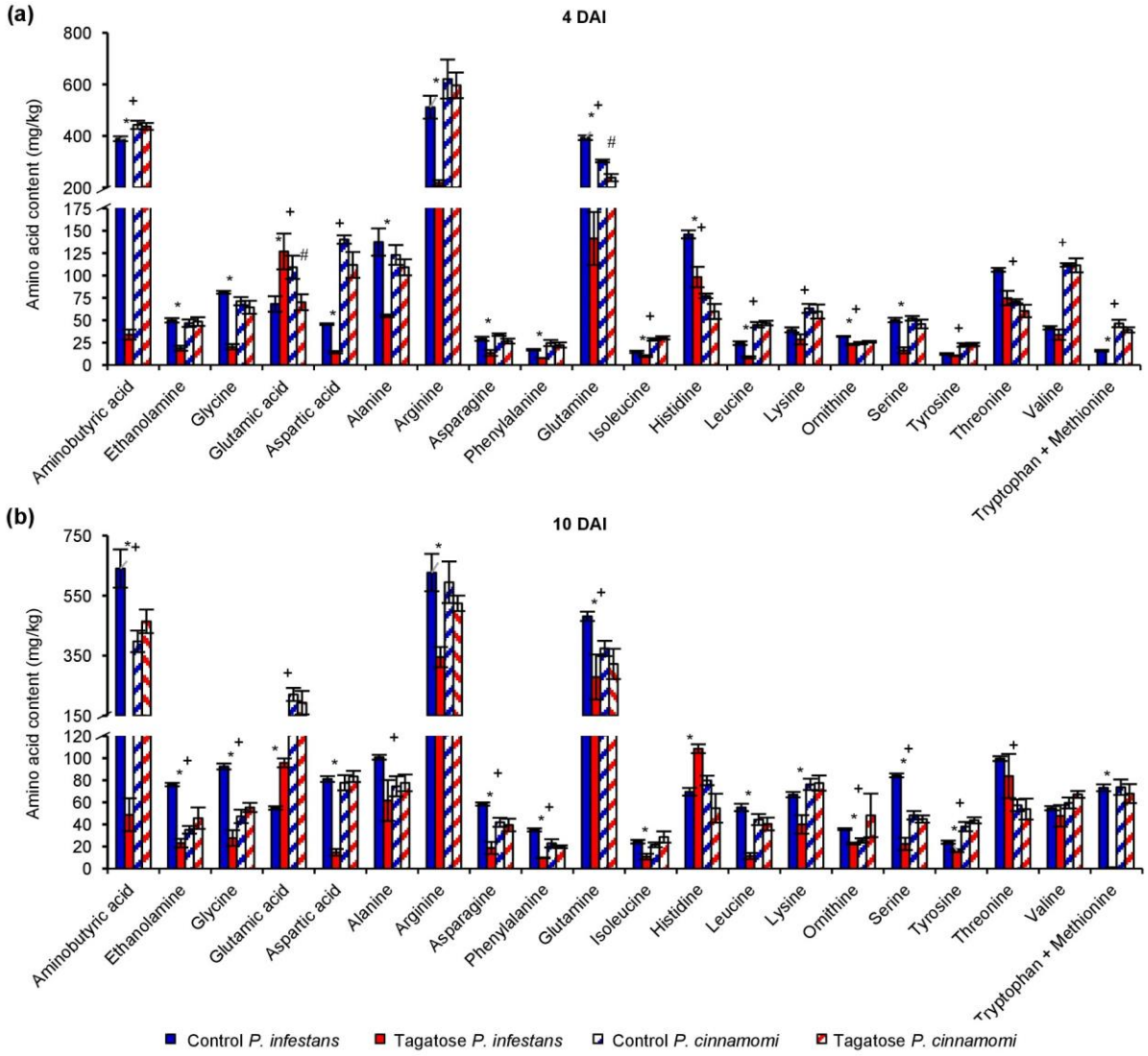

Figure S1. Effect of tagatose on *Phytophthora* spp. amino acid content. *Phytophthora infestans* (solid bars) and *P. cinnamomi* (stripped bars) amino acid content was quantified at four (a) and ten days (b) after incubation (DAI) on pea agar medium in the absence (control, blue) and presence of tagatose (red). The two-way analysis of variance (two-way ANOVA) showed no significant differences between the two experimental repetitions ($P > 0.05$, three replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of six replicates from the two experiments are presented for each treatment. For each amino acid and time point, significant differences between tagatose-incubated samples and control samples are marked with an asterisk (*) for *P. infestans* and a hashtag (#) for *P. cinnamomi*, according to the Student's t-test ($P \leq 0.05$). Significant differences between *P. infestans* control and *P. cinnamomi* control for each amino acidare marked with a plus sign (+), according to the Student's t-test ($P \leq 0.05$). Citrulline was not detected and omitted in the chart.

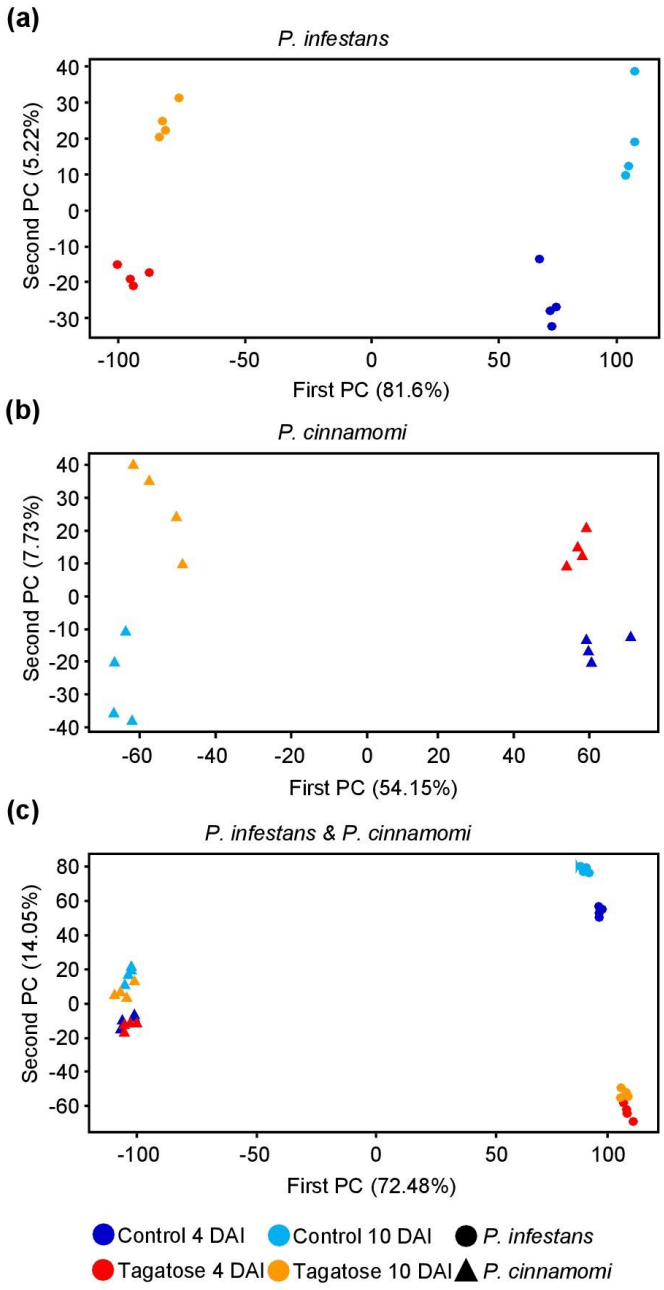

Figure S2. Principal component analysis (PCA) of *Phytophthora* spp. samples. PCA of *Phytophthora infestans* (a), *P. cinnamomi* (b) and *Phytophthora spp.* (c) samples was obtained on Limma-normalised expression values of *P. infestans* (circles) and *P. cinnamomi* (triangles) genes at four and ten days after incubation (DAI) on pea agar medium in the absence (control; blue, 4 DPI and light blue, 10 DAI) and presence of tagatose (red, 4 DPI and orange, 10 DAI) in four replicates. The percentage of variance explained by each principal component (PC) is reported in brackets.

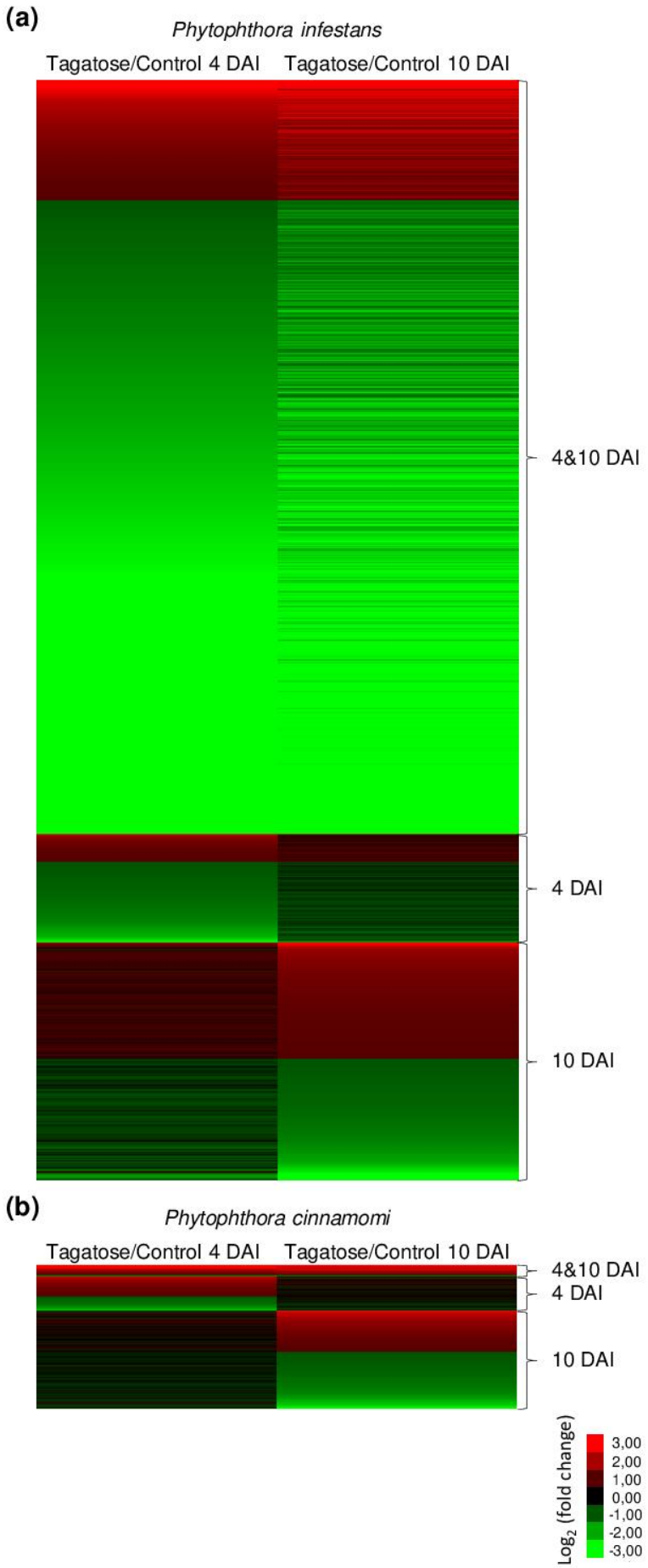
(a)
Phytophthora infestans
Tagatose/Control 4 DAI
Tagatose/Control 10 DAI
4&10 DAI
4 DAI
10 DAI
(b)
Phytophthora cinnamomi
Tagatose/Control 4 DAI
Tagatose/Control 10 DAI
4&10 DAI
4 DAI
10 DAI
Log2 (fold change)
3,00
2,00
1,00
0,00
-1,00
-2,00
-3,00

Figure S3. Clustering of genes modulated by tagatose in *Phytophthora* spp. Heat map diagram of fold change values of differentially expressed gens (DEGs) identified in *Phytophthora infestans* (a) and *P. cinnamomi* (b) at four and ten days after incubation (DAI) on pea agar medium in the presence of tagatose compared to the respective control samples grown in the absence of tagatose. For each *Phytophthora* spp., DEGs were grouped in genes significantly upregulated or downregulated at both time points (4&10 DAI cluster) and exclusively modulated at 4 DAI (4 DAI cluster) or at 10 DAI (10DAI cluster). The heat map diagram was visualised using Java Treeview according to the colour scale legend shown.

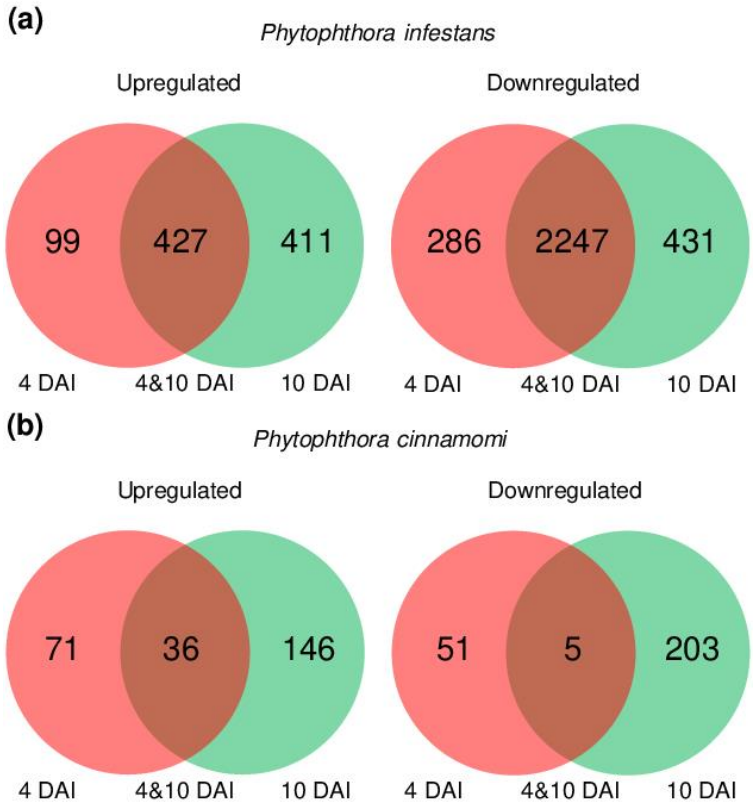

Figure S4. Venn diagrams of genes modulated by tagatose in *Phytophthora* spp. Venn diagrams indicate the distribution of differentially expressed genes (DEGs) identified in *Phytophthora infestans* (a) and *P. cinnamomi* (b) at four and ten days after incubation (DAI) on pea agar medium in the presence of tagatose compared to the respective control samples grown in the absence of tagatose. DEGs of each *Phytophthora* spp. were grouped in genes significantly upregulated or downregulated at both time points (4&10 DAI) and exclusively modulated at 4 DAI or at 10 DAI.

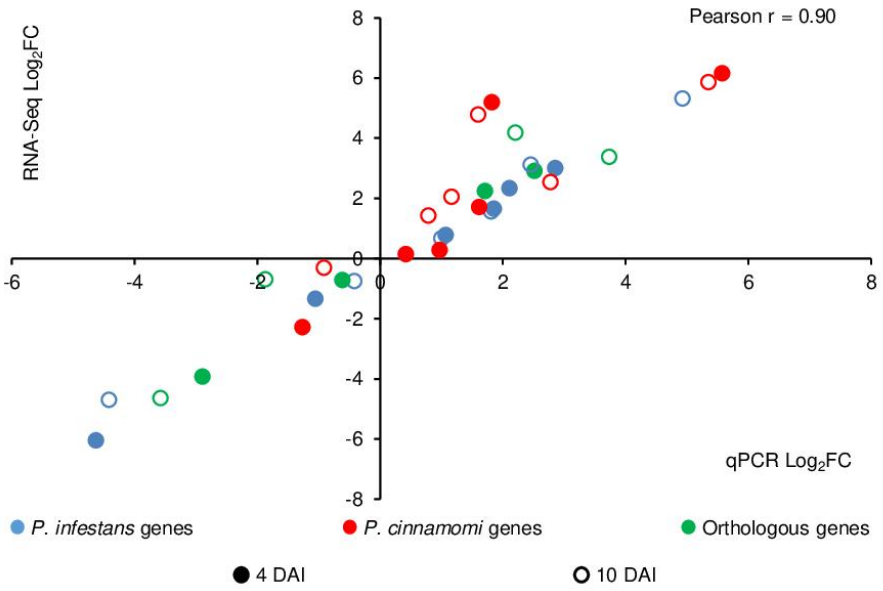

Figure S5. Correlation analysis of gene expression data assessed by RNA-Seq and quantitative real-time PCR. Scatter plot and Pearson correlation value (r) on relative expression levels (Log_2-transformed fold change values) assessed using the RNA-Seq analysis and quantitative real-time PCR (qPCR) analysis of the selected *Phytophthora infestans* (blue) and *P. cinnamomi* (red) and *Phytophthora* orthologous (green) genes (Table S7) at four (solid circle) and ten (open circle) days after incubation (DAI) on pea agar medium in the presence of tagatose calculated as compared to the respective control samples grown in theabsence of tagatose.

TABLE S1 RNA-Seq sequencing and mapping results of each replicate.

TABLE S2 Expression levels of *Phytophthora infestans* genes.

TABLE S3 Expression levels of *Phytophthora cinnamomi* genes.

TABLE S4 Fold change values and functional annotation of genes modulated by tagatose in *Phytophthora infestans*.

TABLE S5 Fold change values and functional annotation of genes modulated by tagatose in *Phytophthora cinnamomi*.

TABLE S6 Expression profiles and functional annotation of *Phytophthora infestans and P. cinnamomi* orthologous genes modulated by tagatose.

TABLE S7 Primer sequences of the *Phytophthora* spp. genes analysed by quantitative real-time PCR.

TABLE S8 Sugar content in the pea agar medium.

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Publication 4: Persistence of the rare sugar tagatose on tomato leaves and its effect on the phyllosphere bacteria (in preparation)

Résumé: Le tagatose est un sucre rare qui inhibe la croissance de plusieurs agents phytopathogènes, tels que *Phytophthora infestans* qui cause le mildiou de la tomate et de la pomme de terre. Le tagatose est un monosaccharide qui ne peut être utilisé comme source de carbone que par certains taxons microbiens. Cependant, les feuilles des plantes (phyllosphère) sont généralement colonisées par une variété de microorganismes, y compris des agents pathogènes des plantes, mais aussi plusieurs microorganismes ayant des effets bénéfiques sur la plante. Par conséquent, le traitement de la plante avec le tagatose l'exposerait à différents microorganismes qui peuvent le dégrader et diminuer son efficacité contre les agents phytopathogènes cibles. Afin d'évaluer la persistance du tagatose sur les feuilles de tomates et son effet sur les bactéries associées, des plantes de tomates ont été traités avec du tagatose, puis les feuilles ont été coupées et lavées pour récupérer les résidus du tagatose et identifier les bactéries de la phyllosphère qui peuvent utiliser le tagatose comme source de carbone. Les traitements avec le tagatose ont augmenté le nombre d'agents de biocontrôle contre les pathogènes de la tomate ainsi que le nombre de bactéries promotrices de croissance des plantes de tomate par rapport au traitement avec l'eau. En particulier, *Bacillus megaterium*, *Microbacterium oxydans* et *Pantoea agglomerans*, précédemment indiqués comme agents de biocontrôle contre les pathogènes de la tomate et aussi comme promoteurs de croissance des plantes de tomate, ont été trouvés dans les suspensions de lavage des feuilles traitées avec le tagatose. De plus, *Pseudomonas brenneri, Enterobacter ludwigii et Pseudomonas putida*, précédemment indiqués comme agents de biocontrôle contre les agents pathogènes de la tomate et aussi comme promoteurs de croissance des plantes de tomate, ont été trouvés dans les suspensions de lavage des feuilles traitées avec une formulation de tagatose. En contrepartie, seul *Pseudomonas putida*, a été trouvé dans les suspensions de lavage des feuilles traitées avec l'eau en tant que bactérie bénéfique. Bien que le tagatose ait été partiellement dégradé par le microbiome de la phyllosphère de la tomate, les résidus de tagatose collectés 1 et 7 jours après le traitement des plantes ont montré une inhibition de la croissance de *P. infestans in vitro*. Par conséquent, le tagatose peut avoir un double effet sur la santé des plantes ; en inhibant les agents pathogènes des plantes (effet direct) et en augmentant le nombre d'agents de biocontrôle qui pourraient contribuer partiellement au contrôle des maladies par compétition pour l'espace, par antagonisme ou *via* l'activation des mécanismes de défense des plantes (effet indirect).

Persistence of the rare sugar tagatose on tomato leaves and its effect on the phyllosphere bacteria

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Abstract: The tagatose is a rare sugar that inhibits the growth of several phytopathogens, such as *Phytophthora infestans* the causal agent of tomato and potato late blight. Tagatose, does not act as a plant resistance inducer but it acts directly on the pathogen by inhibiting sugar metabolism and mitochondrial processes. Therefore tagatose was proposed as promising plant protection product with negligible effects on human health and the environment. However, the treatment of plant with tagatose would also expose it to a diverse population of phyllosphere microbiota that can metabolise tagatose and decrease its efficiency against target phytopathogens. Based on this potential degradation by the phyllosphere microbiota, the aim of this study was to evaluate the persistence of tagatose on tomato leaves and its effect on the associated bacteria in order to provide a deeper knowledge on tagatose efficiency as an eco-friendly fungicide for sustainable plant protection. Although, tagatose was partially degraded by tomato phyllosphere microbiota, tagatose residues collected 1 and 7 days after plant treatment showed an inhibition of *P. infestans* growth *in vitro*. In addition, tagatose treatments affected the diversity of bacterial communities in tomato plant phyllosphere. In particular, tagatose treatments increased the number of potential beneficial bacteria of tomato plants including some biocontrol agents. Therefore tagatose can have a double effect on plant health; by inhibiting plant pathogens (direct effect) and by increasing the number of potential biocontrol agents, which could partially contribute to disease control either by competing for space, antagonism or plant resistance activation (indirect effect).

Keywords: rare sugar, tagatose, phyllosphere bacteria, biological control, *Phytophthora infestans*

1. Introduction

Recently, rare sugars have attracted the attention of the agricultural sector due to their low toxicity and efficacy to control plant diseases caused by a wide range of phytopathogens [1,2]. Rare sugars have been defined as monosaccharides and their derivatives that rarely exist in nature [3]. The potential functional and ecological properties of rare sugars are underestimated due to their limited availability in nature [4]. However, the implementation of novel enzymaticand microbial processes lowered the cost of rare sugar synthesis and extended their use in various industrial and scientific fields, such as agriculture, human nutrition and medicine [4,5]. Among rare sugars, tagatose is a ketohexose that was found at low concentrations in many foods and it was generally recognised 'as safe' by the Food and Drug Administration [6]. It has been reported that tagatose inhibits the growth of several phytopathogens causing crops diseases, such as tomato and potato late blight (*Phytophthora infestans*), grapevine downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*), and cabbage downy mildew (*Hyaloperonospora parasitica*) [2]. Previous studies, showed that tagatose, does not act as a plant resistance inducer but it acts directly on the pathogen by inhibiting sugar metabolism and mitochondrial processes [7-9]. In particular, tagatose caused severe mitochondrial alterations, with the consequent decrease in ATP content and oxygen consumption rate, accumulation of reactive oxygen species (ROS) and the upregulation of genes related to apoptosis and oxidative stress response [7]. The alteration of *P. infestans* mitochondrial processes by tagatose was linked to inhibition of fructokinase and phosphomannose isomerase and β-glucosidase enzymes in *Hyaloperonospora arabidopsidis* and *P. infestans* respectively [8,9]. Therefore, tagatose was proposed as promising plant protection product with negligible effects on human health and the environment [7-9].

In addition to the direct effect against phytopathogens, tagatose could also have an

indirect effect on plant health by acting as a nutritional factor on some indigenous biocontrol microorganisms [10]. In particular, tagatose exhibited a plant prebiotic effect on the phyllosphere microbiota of grapevine and modified the balance of potential pathogenic and potential beneficial microorganisms by selective nutritional and anti-nutritional properties for some specific microbial taxa [10]. Beneficial microorganisms increased by tagatose treatment included *Alternaria* spp., *Aureobasidium* spp., *Exiguobacterium* spp. and *Exophiala* spp. that could partially contribute to disease control by competing for space, antagonism or plant resistance activation [10]. Likewise, it has been shown that tagatose increases the abundance of beneficial bacteria (e.g. *Enterococcus* spp. and *Lactobacillus* spp.) in the human gut microbiota while it impairs the growth of some human bacterial pathogens (e.g. *Streptococcus mutants* and *Salmonella enterica* serovar Typhimurium) [6,11-13]. In fact, tagatose is a monosaccharide that can be used as a carbohydrate source by only certain microbial taxa, such as *Exiguobacterium* spp., *Lactobacillus* spp., and *Lactococcus* spp. [14-17]. On the other hand, tagatose is not catabolised by several human pathogenic bacteria such as *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Yersinia enterocolitica* [18], indicating either its nutritional or antinutritional effects on specific microbial taxa.

Plant leaves (phyllosphere) are usually colonised by a variety of microorganisms including plant pathogens, but also several microorganisms with beneficial effects on the plant [19-21]. Therefore, the treatment of the plant with tagatose would expose it to a wide range of microorganisms that can degrade it and decrease its efficiency against target phytopathogens. Based on the potential degradation of tagatose by the phyllosphere microbiota, the aim of this study was to evaluate the persistence of tagatose on tomato leaves and its effect on the associated bacteria in order to provide deeper knowledge on tagatose efficiency as an eco-friendly fungicide for sustainable plant protection.

2. Materials and Methods

2.1. Phytophthora infestans growth conditions and spore collection

Phytophthora infestans strain VB3 was maintained in glycerol at -80°C in the fungal collection of the Fondazione Edmund Mach, Italy, and they are freely available upon request. *Phytophthora infestans* was grown in Petri dishes on pea agar medium (PAM, 12.5% frozen peas and 1.2% agar in distilled water) at 18 ± 1°C [7]. Seven days-old *P. infestans* dishes were used for sporangia collection. Briefly, plates of *P. infestans* were filled with 2 mL pea broth (PB, 12.5% frozen peas in distilled water), sporangia were scraped with a sterile spatula and the suspension was filtered using a sterile Pasteur pipette containing a fine mesh. The concentration of sporangia was determined with a haemocytometer and was adjusted to a final concentration of 10^4 sporangia/mL. The stock solution (50 g/L in distilled water) of tagatose (Bi-PA, Londerzeel, Belgium) was filter sterilised and used at a final concentration of 5 g/L for all experiments.

2.2. Plant treatment and leaf collection

Four weeks old tomato plants (*Solanum Lycopersicum* var. Moneymaker) were purchased and grown in plastic tunnel greenhouse. After two weeks, healthy tomato plants were treated with a water solution of 5 g/L pure tagatose (TAG) or a tagatose formulation containing 5 g/L tagatose (F_TAG; wettable powder containing 80% tagatose w/w; Bi-PA nv, Londerzeel, Belgium) on all leaves using a compressed air hand sprayer (20–30 mL for each plant). As control, plants were treated with distilled water (H_2O). Leaf washing was carried out as reported by Cappelletti et al. [22]. For each treatment, asymptomatic leaves were randomly collected 1 and 7 days post treatment (dpt) and three, replicates of 50 leaves were obtained from twenty plants for each treatment. Leaves were placed in sterile plastic boxes and washed with 100 mL of isotonic solution (0.85% sodium chloride with 0.01%

Tween 20) by manual shaking for 15 minutes. Leaf washing suspensions were filtered with sterile cheesecloth, and 100 mL of suspension for each replicate was used for bacterial isolation. The remaining part was filter sterilised and incubated at -20 °C for tagatose quantification and efficacy tests against *P. infestans in vitro*.

2.3. Tagatose quantification

Tagatose quantification in each leaf washing suspension (aliquot of 50 mL) resulted from H_2O-, TAG- and F_TAG- treated plants, tagatose concentration was assessed by ion chromatography (Chemistry Unit at Fondazione Edmund Mach) as reported in Perazzolli et al. [10], and it was converted as tagatose residues per unit of fresh leaf weight (mg/g) using a calibration curve of 98.5 % pure tagatose (Sigma-Aldrich, St.Louis, MO, USA) dissolved in ultra-pure water within a range between 2 and 40 µg/mL. Briefly, samples were diluted 50 fold in ultrapure water, filtered through a 0.45µm PTFE membrane (Sartorius, Goettingen, Germany) and analysed with an ionic chromatograph ICS 5000 (Dionex-Thermo Scientific, Waltham, MA, USA), equipped with anautosampler, a quaternary gradient pump, a column oven and a pulsed amperometric detector with a gold working electrode and a palladium counter electrode. The separation was obtained by injecting 5 µL of diluted sample onto a CarboPac PA200 3 × 250 mm analytical column (Dionex-Thermo Scientific, Waltham, MA, USA), preceded by a CarboPac PA200 3 × 50 mm guard column (Dionex- Thermo Scientific), with a KOH gradient (from 1 to 100 mM) at 0.4 mL/ min flow rate.

2.4. Isolation of culturable bacteria

One millilitre of each leaf washing suspension was 10-fold serially diluted, and 0.1 mL of each dilution was plated on nutrient agar (NA) to quantify total culturable bacteria. A minimal medium agar (containing per litter: K_2HPO_4 0.4 g, KH_2PO_4 0.4 g, $(NH_4)2PO_4$ 0.4 g,

NaCl 0.3 g, 1 mL of micronutrients solution, 1 mL of vitamins solution and 1.5% agar) [23] amended with 5 g/L of tagatose as unique carbon source was used to isolate tagatose-degrading bacteria. After incubation at 25 °C for 48 h, colony forming units (CFUs) per mL of leaf washing suspension were determined. Bacterial representative isolates were selected visually for each treatment based on morphological analysis of bacterial colonies for further identification by 16S sequencing.

2.5. Identification of bacterial isolates

Molecular characterisation of tagatose-degrading bacterial isolates from leaf washing suspensions of H_2O, TAG- and F_TAG-treated plants was performed by using 16S rRNA gene sequences as described by Esmaeel et al. [24]. Briefly, bacteria isolated from minimal medium amended with tagatose were grown in LB broth on a rotary shaker (160 rpm) at 30 °C for 18 h. Total DNA was extracted using the Wizard Genomic Purification DNA Kit (Promega Corp., Madison, WI, USA). The universal 16S rRNA primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RP2 (5'-TACGG CTACCTTGTTACGACTT- 3') were used. PCR Master Mix (2X) (Thermo Scientific Fermentas, Villebon sur Yvette, France) was used as a mixture of Taq DNA polymerase 0.05 units/ µL, reaction buffer, 4 mM MgCl2 and 0.4 mM of each dNTPs. The total volume of each reaction was 50 µL and contained 25 µL of master mix, 17.5 µL of sterile nuclease free H_2O, 1.25 µL of each primer (0.5 pmol/ µL final concentration) and 5 µL of DNA (10 to 40 ng/ µL). PCR was carried out in a DNA Thermocycler (C1000 touch thermal cycler,Bio-Rad, Hercules, CA, USA) with the following thermal cycles: one cycle at 94 °C for 5 min; 30 cycles (94 °C, 1 min; 56 °C, 30 s; 72 °C, 2 min), and a final extension at 72 °C for 10 min. PCR products were evaluated by agarose gel. The resulting PCR product sizes, ranged from 1450 to 1500 bp, were purified with the GeneJET Gel Extraction Kit (Thermo Scientific Fermentas, Waltham, USA). Purified PCR

product was sequenced by express single sequencing service of GENWIZ Co., Ltd. (Leipzig, Germany) using Sanger sequencing technology. The sequencing reaction was performed in two directions using the forward and reverse primers (FD2 and RP1) to cover the length of the 16S rRNA gene. After trimming, forward and reverse sequences were aligned and then assembled to obtain the full contiguous sequence. Then, the sequences were submitted to blast basic local alignment search tool (BLAST) provided online by the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The nearest 16S rRNA gene sequences were downloaded and the determination of phylogenic relationships was performed by the program phylogenic analysis mega [25]. The tree was built using the neighbour-joining method [26]. The evolutionary distances were computed using the maximum composite likelihood method [27]. The rate variation among sites was modelled with a gamma distribution with five rate categories. Bootstrap analysis with 1000 replicates was performed to assess the support of the clusters [28]. All sequences were submitted to the GenBank database [accession numbers: MW689284 - MW689301].

2.6. Evaluation of the effect of tagatose residues on P. infestans growth

Growth of *P. infestans* was assessed by incubating sporangia in PB amended with water (CTR(-), negative control) and leaf washing suspensions of TAG- and F_TAG-treated plants (collected 1 and 7 dpt). *Phytophthora infestans* suspensions contained 10^4 sporangia/mL were dispensed in 200-µL final volume into a 96-well microplate and the plate was incubated under orbital shaking at 80 rpm at 18 ± 1°C. As positive control treatments, tagatose (5 g/L) and copper (10 g/L) were used. Growth of *P. infestans* was monitored by measuring the absorbance (620 nm) at 0, 36 and 72 h of incubation time using a TECAN Microplate Reader (Infinite F200 Pro Luminometer, Männedorf, Switzerland). Three replicates (wells) were assessed for each treatment and the experiment was carried out twice. For microscopic analysis,

P. infestans sporangia were incubated in PB amended with water (CTR (-), negative control) and leaf washing suspensions of TAG- and F_TAG-treated plants (collected 1 and 7 dpt). For microscopic observations, *P. infestans* suspensions contained 10^4 sporangia/mL were dispensed in 500- µL final volume into a 24-well microplate and the plate was incubated under orbital shaking at 80 rpm at 18 ± 1°C for 72 h. As positive control treatments, tagatose (5 g/L) and copper (10 g/L) were used. After 72 h of incubation, microscopic observations were carried out with an Invitrogen™ EVOS™ FL Auto Imaging System (Thermo Fisher Scientific, Wilmington, DE, United States). Two replicates were analysed for each treatment and the experiment was carried out twice.

2.7. Statistical Analysis

All experiments were carried out twice and data were analysed with Statistica 13.1 software (Dell, Round Rock, TX, United States). Normal distribution (Kolmogorov–Smirnov test, $P > 0.05$) and variance homogeneity of the data (Levene's tests, $P > 0.05$) were checked and parametric tests were used when both assumptions were respected. Each experimental repetition was analysed singularly and a factorial analysis of variance (factorial ANOVA) was used to demonstrate non-significant differences between the two experiments ($P > 0.05$). Data from the two experimental repetitions were pooled and significant differences were assessed with the Student's t-test ($P \leq 0.05$) or Tukey's test ($P \leq 0.05$) in case of pairwise or multiple comparisons, respectively.

3. Results and discussion

3.1. Tagatose is partially degraded by tomato phyllosphere microbiota

The quantification of tagatose residues present in leaf washing suspensions of TAG and F_TAG-treated plants 1 and 7 dpt showed a partial tagatose degradation of 75.22% and

78.01% between the two time points in leaf washing suspensions of TAG and F_TAG- treated plants respectively (Figure 1). These results showed that tagatose formulation (F_TAG) did not protect the active ingredient from degradation by the phyllosphere microbiota. As expected, tagatose was found in very small amount in leaf washing suspensions of H_2O treated plants with no significant differences between the two time points (Figure 1).

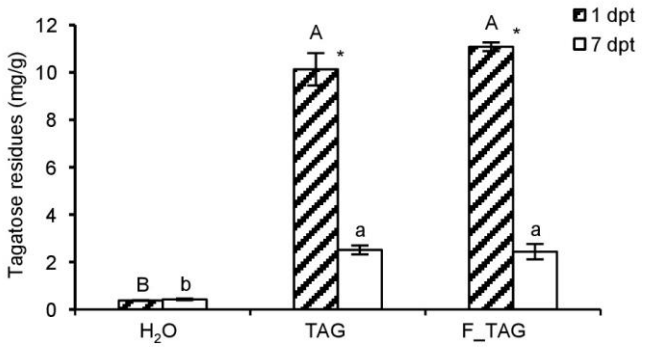


Figure 1. Quantification of tagatose residues on tomato leaves. Tomato plants were treated with distilled water (H_2O), 5 g/L tagatose (TAG) or a formulation containing 5 g/L tagatose (F_TAG). Tagatose was assessed in leaf washing suspensions of H_2O-, TAG- and F_TAG -treated plants collected at one (stripped bars) and seven days (clear bars) post treatment (dpt) and expressed per unit of leaf fresh weight (mg/g). The two-way analysis of variance (two-way ANOVA) showed no significant differences between the two experimental repetitions ($P > 0.05$, three replicates per experiment) and data from the two experiments were pooled. Mean and standard error values of six replicates from the two experiments are presented for each treatment. Different uppercase and lowercase letters indicate significant differences among leaf washing suspensions collected at one and seven days post treatments respectively according to Tukey's test ($P \leq 0.05$). For each treatment, significant differences between one and seven days post treatments are marked with an asterisk (*) according to the Student's t- test ($P \leq 0.05$).

The number of culturable tomato phyllosphere bacteria (Log10 CFU/mL) was not affected by the different treatments at both time points (Figure S1). Likewise, tagatose treatment did not affect the number of culturable grapevines bacteria per leaf unit under field conditions [10]. Since tagatose can be utilised as a carbohydrate source by only certain

microbial taxa [14-17] only some bacteria from the different leaf washing suspensions were able to grow on minimal medium amended with tagatose as unique carbon source indicating its nutritional or antinutritional effects on specific microbial taxa (Figures S1 and S2).

3.2. Tagatose affected the diversity of bacterial communities of tomato phyllosphere under greenhouse conditions

The molecular identification of isolates from leaf washing suspensions of H_2O, TAG and F_TAG-treated plants showed different bacterial communities among the treatments (Figures 2, 3 and 4 and Table 1).

Table 1. List of tagatose-degrading bacteria isolated from the different leaf washing suspensions. Tagatose-degrading bacteria isolated from leaf washing suspensions of tomato plants treated with distilled water (H_2O), 5 g/L tagatose (TAG) or a formulation containing 5 g/L tagatose (F_TAG) were identified using 16S rRNA gene sequences. Isolate code (column A), treatment (column B), bacterial genus (column C), the best hit bacterial specie and the respective percentage of identity (column D-E) and the accession number (F) are reported.

Isolate code	Treatment	Bacteria	Best hit bacteria	Per. identity	Accession number
TAG_25	H_2O	*Acinetobacter* sp.	*Acinetobacter parvus*	99.25%	MW689301
TAG_5/9	H_2O	*Pseudomonas* sp.	*Pseudomonas putida*	99.85%	MW689286
TAG_6/14	H_2O	*Acinetobacter* sp.	*Acinetobacter johnsonii*	99.92%	MW689287
TAG_20	H_2O	*Plantibacter* sp.	*Plantibacter flavus*	99.58%	MW689296
TAG_21/18	H_2O	*Methylobacterium* sp.	*Methylobacterium radiotolerans*	99.92%	MW689297
TAG_12	H_2O	*Sphingomonas* sp.	*Sphingomonas kyungheensis*	96.75%	MW689292
TAG_1	TAG	*Bacillus* sp.	*Bacillus megaterium*	99.88%	MW689284
TAG_16	TAG	*Microbacterium* sp.	*Microbacterium oxydans*	99.46%	MW689295
TAG_21/18	TAG	*Methylobacterium* sp.	*Methylobacterium radiotolerans*	99.21%	MW689297
TAG_10/19	TAG	*Sphingomonas* sp.	*Sphingomonas paucimobilis*	100%	MW689290
TAG_11	TAG	*Pantoea* sp.	*Pantoea agglomerans*	98.88%	MW689291
TAG_22	TAG	*Sphingobium* sp.	*Sphingobium yanoikuyae*	99.69%	MW689298
TAG_23	TAG	*Comamonas* sp.	*Comamonas testosteroni*	99.39%	MW689299
TAG_13	TAG	*Ralstonia* sp.	*Ralstonia insidiosa*	99.63%	MW689293
TAG_6/14	TAG	*Acinetobacter* sp.	*Acinetobacter johnsonii*	99.39%	MW689287
TAG_15	TAG	*Rhodococcus* sp.	*Rhodococcus cercidiphylli*	99.59%	MW689294
TAG_4	F_TAG	*Pseudomonas* sp.	*Pseudomonas brenneri*	99.71%	MW689285
TAG_10/19	F_TAG	*Sphingomonas* sp.	*Sphingomonas paucimobilis*	99.71%	MW689290
TAG_7	F_TAG	*Acinetobacter* sp.	*Acinetobacter haemolyticus*	98.94%	MW689288
TAG_8	F_TAG	*Enterobacter* sp.	*Enterobacter ludwigii*	99.54%	MW689289
TAG_5/9	F_TAG	*Pseudomonas* sp.	*Pseudomonas putida*	99.90%	MW689286
TAG_24	F_TAG	*Microbacterium* sp.	*Microbacterium phyllosphaerae*	99.62%	MW689300

In particular, *Acinetobacter parvus*, *Pseudomonas putida*, *Acinetobacter johnsonii*, *Plantibacter flavus*, *Methylobacterium radiotolerans* and *Sphingomonas kyungheensis* were isolated from leaf washing suspensions of H_2O-treated plants (Figure 2).

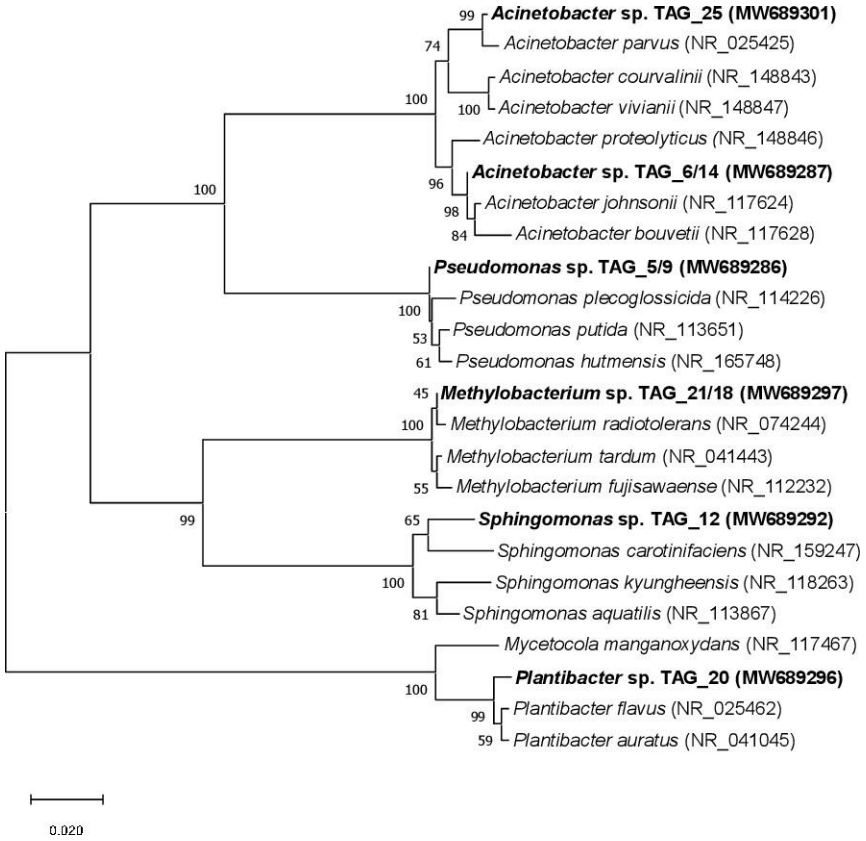


Figure 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of tagatose-degrading bacterial isolates from leaf washing suspensions of tomato plants treated with distilled water (H_2O). The identified bacteria are written in bold and accession numbers of all bacteria are indicated between brackets. The analysis was conducted with Kimura's two-parameter calculation model in MEGA version 6.0. Bootstrap values out of 1000 are shown at nodes. The scale bar represents the number of substitutions per site. Sequences were submitted to the GenBank database [accession numbers: MW689301, MW689286, MW689287,MW689296, MW689297, MW689292]

However, *Bacillus megaterium, Microbacterium oxydans, Methylobacterium radiotolerans, Sphingomonas paucimobilis, Pantoea agglomerans, Sphingobium yanoikuyae, Comamonas testosteroni, Ralstonia insidiosa, Acinetobacter johnsonii and Rhodococcus cercidiphylli* were isolated from leaf washing suspensions of TAG-treated plants (Figure 3).

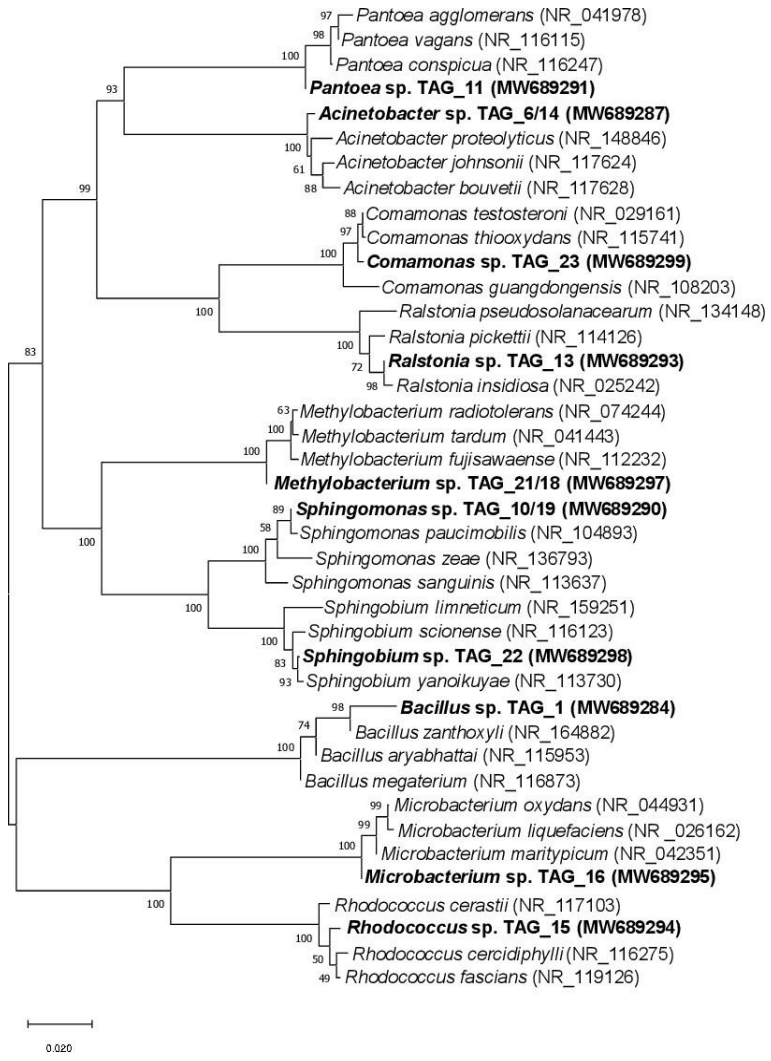


Figure 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of tagatose-degrading bacterial isolates from leaf washing suspensions of tomato plants treated with 5 g/L tagatose (TAG). The identified bacteria are written in bold and accession numbers of all bacteria are indicated between brackets. The analysis was conducted with Kimura's two-parameter calculation model in MEGA version 6.0. Bootstrap values out of 1000 are shown at nodes. The scale bar represents the number of substitutions per site. Sequences were

submitted to the GenBank database [accession numbers: MW689284, MW689295, MW689297,MW689290, MW689291, MW689298, MW689299, MW689293, MW689287, MW689294]

Moreover, *Pseudomonas brenneri*, *Sphingomonas paucimobilis*, *Acinetobacter haemolyticus*, *Enterobacter ludwigii*, *Pseudomonas putida* and *Microbacterium phyllosphaerae* were isolated from leaf washing suspensions of F_TAG-treated plants (Figure 4).

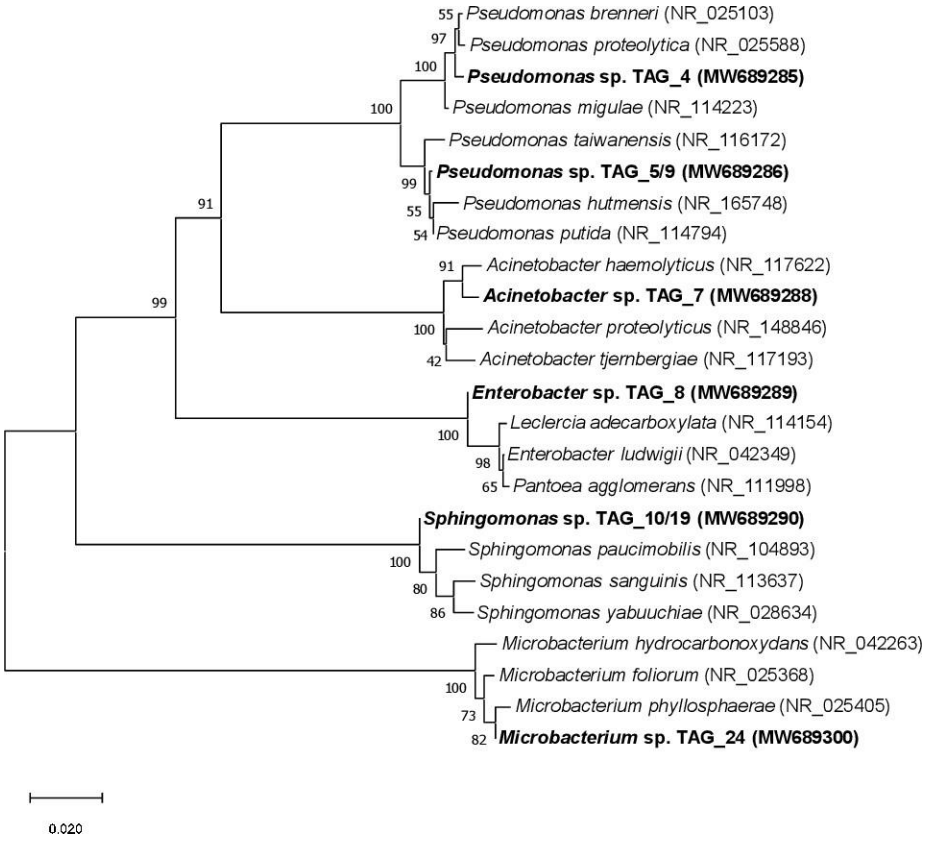


Figure 4. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of tagatose-degrading bacterial isolates from leaf washing suspensions of tomato plants treated with a formulation containing 5 g/L tagatose (F_TAG). The identified bacteria are written in bold and accession numbers of all bacteria are indicated between brackets. The analysis was conducted with Kimura's two-parameter calculation model in MEGA version 6.0. Bootstrap values out of 1000 are shown at nodes. The scale bar represents the number of substitutions

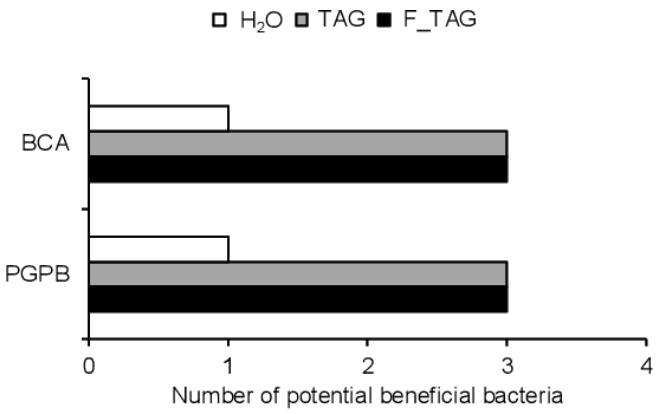
per site. Sequences were submitted to the GenBank database [accession numbers: MW689285, MW689290, MW689288, MW689289, MW689286, MW689300]

Similarly, previous studies showed that tagatose treatment changed the structure of the grapevine leaf microbiota [10] as well as the composition of gut microbiota [29]. Moreover, it has been shown that other rare sugars such as xylitol can shift the structure of the oral microbial community [30] indicating that rare sugars can impact the microbiome diversity in different environments.

The present study indicated for the first time that *Plantibacter* spp., *Sphingomonas* spp., *Microbacterium* spp., *Ralstonia* spp., *Rhodococcus* spp. and *Enterobacter* spp. can use tagatose as carbon source. On the other hand, the rest of genera have been previously reported to metabolise tagatose as unique carbon source. For example, it has been found that *Acinetobacter* spp. can metabolise tagatose using L-ribose isomerase [31] while *Pseudomonas* spp. can produce sorbose from tagatose using D-tagatose 3-epimerase [32].

3.3. Tagatose increased the number of potential beneficial bacteria on tomato leaves

Tagatose treatments (TAG and F_TAG) increased the number of biocontrol agents against tomato pathogens (BCA) and the number of tomato plant growth promoting bacteria (PGPB) compared with H_2O treatment (Figure 5).



BCA/PGPB	H_2O	*Pseudomonas putida*
	TAG	*Bacillus megaterium* *Microbacterium oxydans* *Pantoea agglomerans*
	F_TAG	*Pseudomonas brenneri* *Enterobacter ludwigii* *Pseudomonas putida*

Figure 5. Abundance of potential beneficial bacteria in leaf washing suspensions. Tomato treatment with 5 g/L tagatose (TAG, grey) and a formulation containing 5 g/L tagatose (F_TAG, black) increased the number of biocontrol agents against tomato pathogens (BCA) and the number of tomato plant growth promoting bacteria (PGPB) compared with the treatment with distilled water (H_2O, white). Potential beneficial bacteria from different leaf washing suspensions were determined according to the previous literature.

In particular, *Bacillus megaterium*, *Microbacterium oxydans* and *Pantoea agglomerans* previously reported as both BCA against tomato pathogens [33-36] and tomato PGPB [37-39] were found in leaf washing suspensions of TAG-treated plants. Moreover, *Pseudomonas brenneri*, *Enterobacter ludwigii* and *P. putida* previously reported as both BCA against tomato pathogens [40,41] and tomato PGPB [42-44] were found in leaf washing suspensions of F_TAG-treated plants while only *Pseudomonas putida* as potential beneficial bacteria was found in leaf washing suspensions of H_2O-treated plants. Similarly, tagatose treatment affected the diversity and taxonomic structure of leaf bacterial populations and increased the relative abundance of bacterial genera comprising potential biocontrol agents against

grapevine pathogens such as *Exiguobacterium* spp. under field conditions [10]. These beneficial bacteria could partially contribute to disease control by competing for space, antagonism or plant resistance activation [10]. Accordingly, tagatose was found to increase the abundance of beneficial bacteria (*Enterococcus* spp. and *Lactobacillus* spp.) in the human gut microbiota [6].

3.4. Tagatose residues inhibited the growth of P. infestans in vitro

In order to evaluate whether tagatose residues still have an activity against *P. infestans*, sporangia were incubated in the presence of leaf washing suspensions of TAG and F_TAG-treated plants. *Phytophthora infestans* growth was inhibited after 72h of incubation with leaf washing suspensions of TAG and F_TAG-treated plants collected at 1 and 7 dpt and the growth was intermediate between that of negative control samples (CTR (-)) and samples incubated with 5 g/L tagatose and 10 g/L copper (Figure 6).

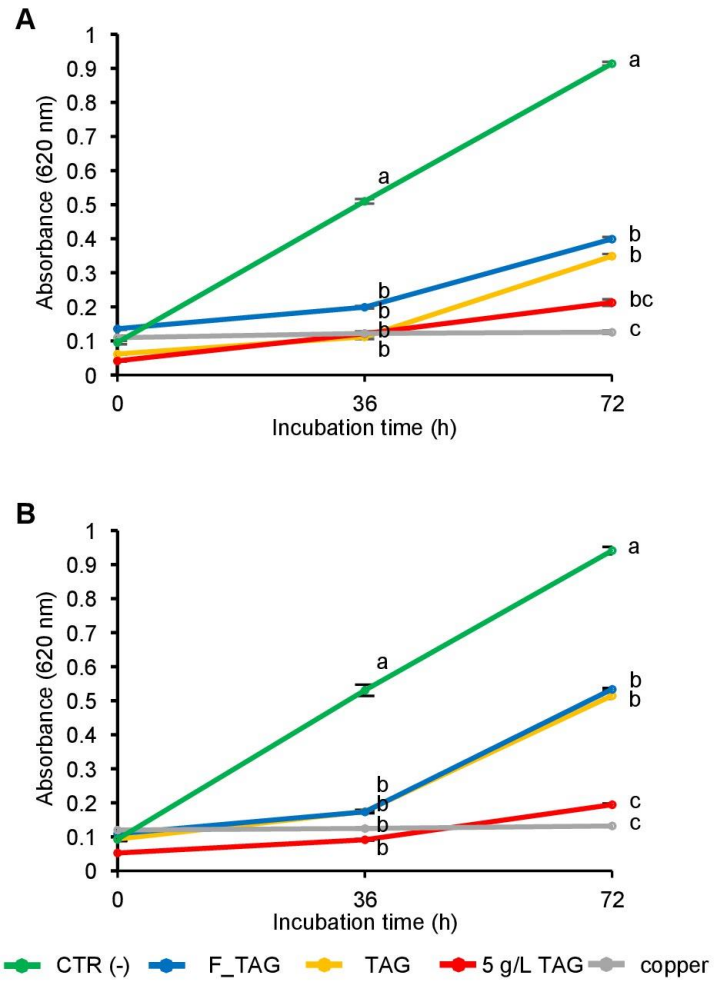


Figure 6. *Phytophthora infestans* growth inhibition by leaf washing suspensions. Tomato plants were treated with distilled water (H_2O), 5 g/L tagatose (TAG) or a formulation containing 5 g/L tagatose (F_TAG) The effect of leaf washing suspensions collected at one **(A)** and seven days **(B)** post treatment on *Phytophthora infestans* growth was evaluated by incubating *P. infestans* sporangia in pea broth amended with water (CTR (-), green), leaf washing suspension of plants treated with 5 g/L tagatose (TAG, orange) and leaf washing suspension of plants treated with a formulation containing 5 g/L tagatose (F_TAG, blue). Tagatose (5 g/L, red) and copper (10 g/L, grey) were used as positive control treatments. The absorbance (620 nm) was assessed at 0, 36 and 72 h of incubation time. The two-way analysis of variance (two-way ANOVA) showed no significant differences between the two experimental repetitions ($P > 0.05$, three replicates per experiment) anddata from the two experiments were pooled. Mean and standard error values of six replicates from the two experiments are presented for each treatment. For each time point, different letters indicate significant differences among the treatments according to Tukey's test ($P \leq 0.05$). No differences were found among treatments at 0 h of incubation, according to Tukey's test ($P \leq 0.05$)

To further validate the effect of leaf washing suspensions of TAG and F_TAG-treated plants on *P. infestans* growth, microscopic analyses were carried out. The growth inhibition of *P. infestans* after 72 h of incubation with leaf washing suspensions of TAG and F_TAG-treated plants collected at 1 and 7 dpt was intermediate between that of negative control samples (CTR (-)) and samples incubated with 5 g/L tagatose and 10 g/L copper (Figures 7 and S3). In line with the decrease of tagatose residues measured at 1 and 7 dpt (Figure 1), the growth inhibition of *P. infestans* by leaf washing suspensions of TAG and F_TAG-treated plants 7 dpt was lower than the growth inhibition by leaf washing suspensions of TAG and F_TAG-treated plants 1 dpt (Figure 7).

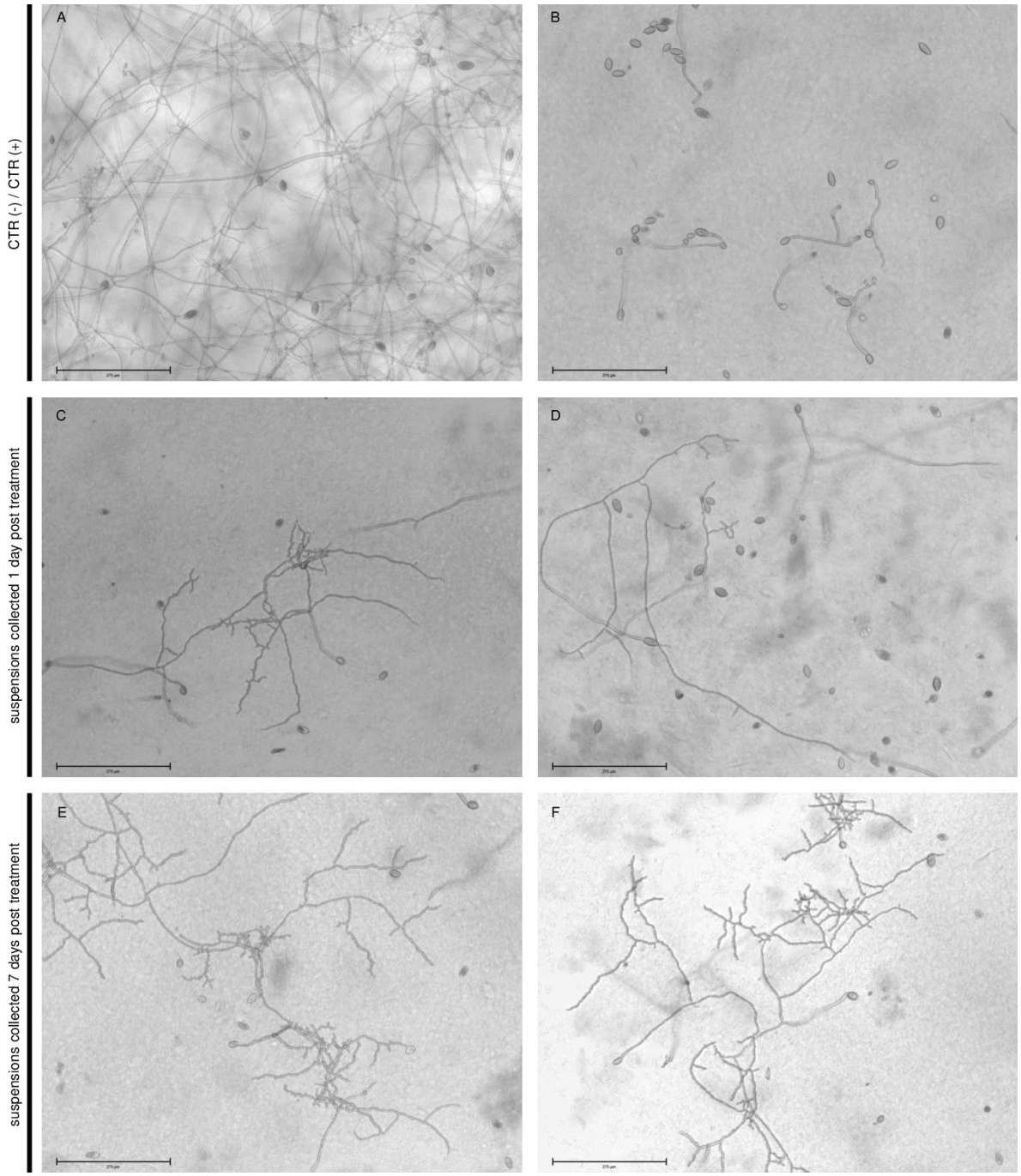


Figure 7. Microscopy observations of *Phytophthora infestans* treated with leaf washing suspensions of tomato plants. Microscopy observations of *P. infestans* sporangia incubated in pea broth amended with water (CTR (-), **A**), 5 g/L tagatose (CTR (+), **B**), leaf washing suspension of plants treated with 5 g/L tagatose (**C**: suspension collected one day post treatment, **E**: suspension collected seven days post treatment) and leaf washing suspensions of tomato plants treated with a formulation containing 5 g/L tagatose (**D**: suspension collected one day post treatment, **F**: suspension collected seven days post treatment) after 72 h of incubation. Two replicates were analysed foreach treatment and the experiment was carried out twice with similar results. Bars correspond to 275 µm.

Although, tagatose was partially degraded by the phyllosphere microbiota, these experiments indicated that tagatose residues in leaf washing suspensions of TAG and F_TAG-treated plants can inhibit *P. infestans*. Accordingly, Mochizuki et al. [9] reported that tagatose had a 7-day residual effect and that applications at 7-day intervals would be practical. For instance low concentration of tagatose (2.5 mM = 0.45 g/L) significantly inhibited conidiation of the oomycete *Hyaloperonospora arabidopsidis* on *A. thaliana* seedlings with four leaves [9]. Similarly tagatose at low concentration (0.8 g/L) showed a significant reduction of downy mildew severity caused by *Plasmopara viticola* under greenhouse conditions [10] indicating that low concentrations of tagatose can still inhibit oomycete development.

4. Conclusions

Although, tagatose was partially degraded by tomato phyllosphere microbiota, the tagatose residues collected 1 and 7 days after plant treatment showed an inhibition of *P. infestans* growth *in vitro*. In addition tagatose affected the composition of the phyllosphere bacteria and increased the number of biocontrol agents against tomato pathogens (BCA) and the number of tomato plant growth promoting bacteria (PGPB). Therefore, the tagatose might have a double effect on plant health; by inhibiting plant pathogens (direct effect) and by increasing the number of potential biocontrol agents which could partially contribute to disease control by competing for space, antagonism or plant resistance activation (indirect effect). Further High-throughput metagenomic analysis of plant microbial communities shift after tagatose application is required in order to better understand the multiple uses of this rare sugar in agriculture.

Supplementary Materials

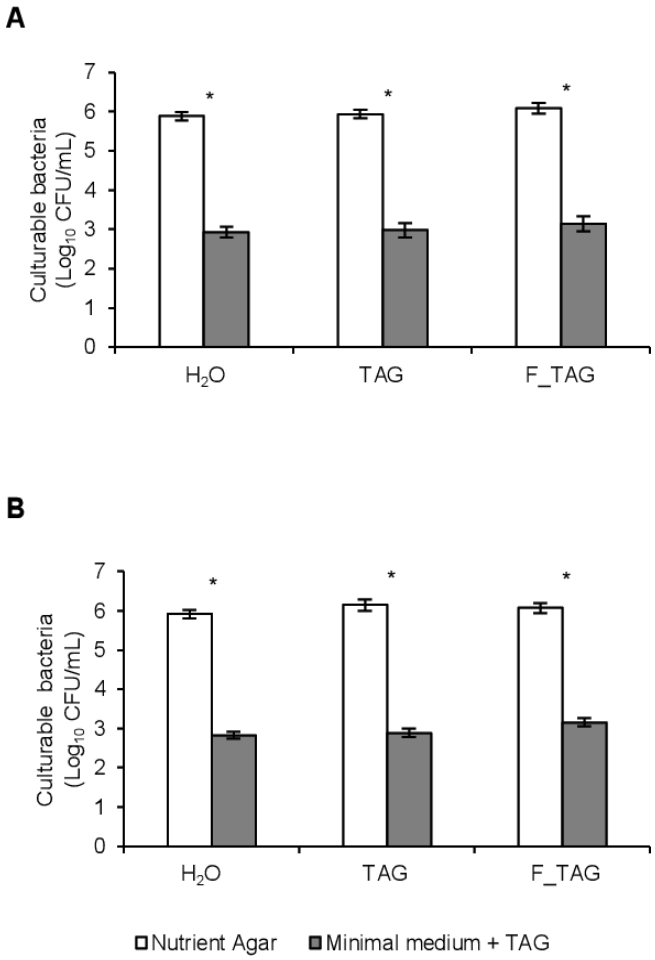


Figure S1. Effect of plant treatments on culturable bacteria of tomato leaves. Bacterial colony forming units (CFU) isolated from leaf washing suspensions (CFU/mL) of tomato plants treated with distilled water (H_2O), 5 g/L tagatose (TAG) or a formulation containing 5 g/L tagatose (F_TAG) collected at one **(A)** and 7 days **(B)** post treatment were assessed by dilution plating in nutrient agar (clear bars) and minimal medium amended with tagatose as unique carbon source (grey bars). The two-way analysis of variance (two-way ANOVA) showed no significant differences between the two experimental repetitions ($P > 0.05$, three replicates per experiment) and data from the two experiments were pooled. Mean and standard errors Log_{10}-transformed values from six replicates from the two experiments are presented for each treatment. For each treatment significant differences between bacterial CFUs on nutrient agar and on minimal medium amended with tagatose are marked with an asterisk (*) according to the Student's t-test ($P \leq 0.05$).

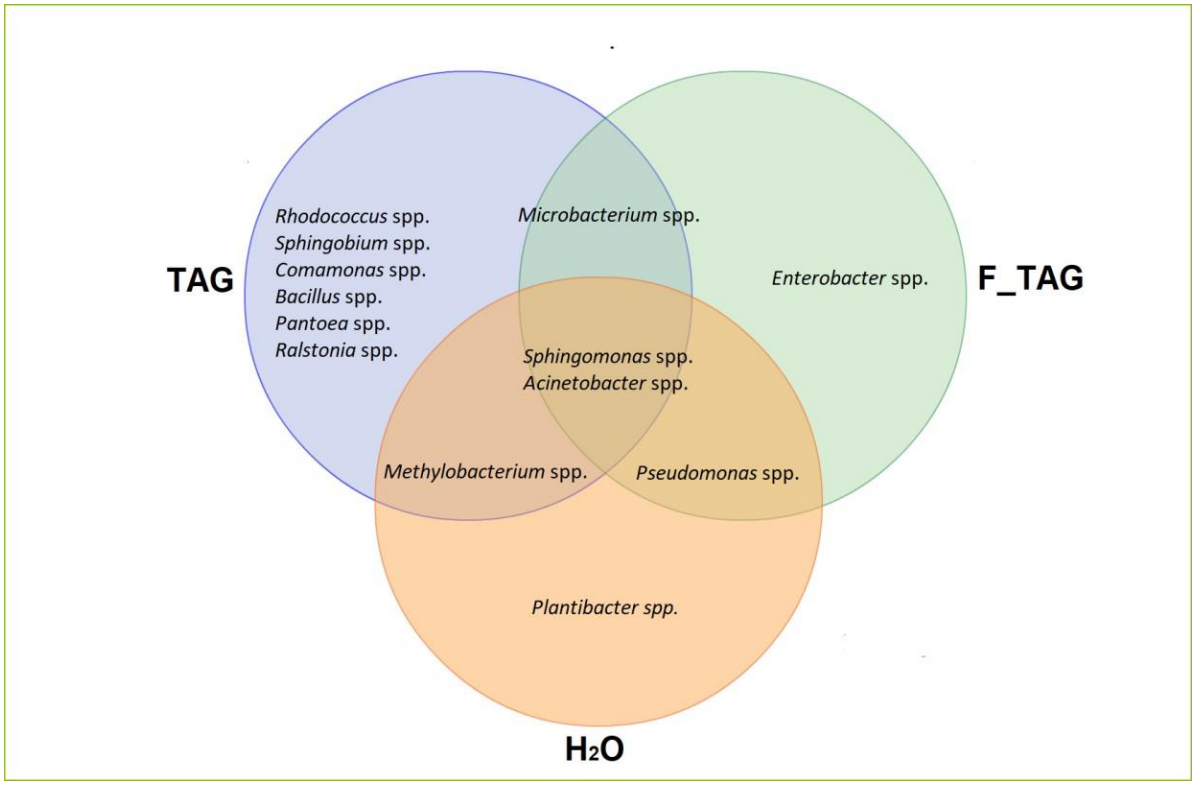


Figure S2. Venn diagram of tagatose-degrading bacterial genera isolated from tomato leaves. Venn diagram indicates the distribution of tagatose-degrading bacterial genera from leaf washing suspensions of tomato plant treated with water (H2O), 5 g/L tagatose (TAG) or a formulation containing 5 g/L tagatose (F_TAG).

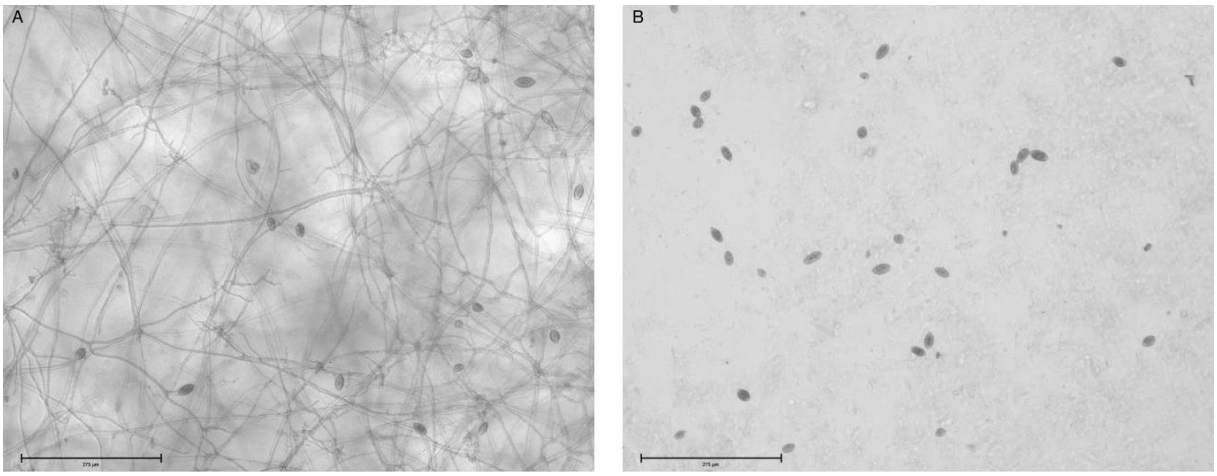


Figure S3. Microscopy observations of *Phytophthora infestans* treated with water and copper. Microscopy observations of *P. infestans* sporangia incubated in pea broth amended with water (CTR (-), A) and 10 g/L copper (CTR (+), B) after 72 h of incubation. Two replicates were analysed for each treatment and the experiment was carried out twice with similar results. Bars correspond to 275 um.

Conflicts of Interest: AC and AN were employed by Bi-PA nv. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CONCLUSION AND FUTURE PERSPECTIVES

Tagatose inhibited *P. infestans* growth *in vitro* and led to severe mitochondrial alterations, with the formation of circular and concentric cristae. Moreover, the ATP content and oxygen consumption rate were decreased, while the ROS generation and expression of apoptosis- and oxidative stress-related genes were increased, suggesting the development of severe deficiencies in the mitochondrial processes of tagatose-incubated *P. infestans*. Conversely, *P. cinnamomi* growth and mitochondrial ultrastructure were only slightly impacted by tagatose with no significant effects on mitochondrial activities, indicating species-specific responses to this rare sugar. The opposite response of *P. infestans* and *P. cinnamomi* to tagatose incubation can be ascribed to their different lifestyles manifested by difference in hosts (*Solanum* species *vs* annual and herbaceous perennial species), infection sites (shoots *vs* roots) and host ranges (narrow *vs* broad). Therefore, further studies are necessary to unravel the link between the different lifestyles and the different responses to this rare sugar.

The different response of *P. infestans* and *P. cinnamomi* to tagatose incubation was linked to species-specific metabolic and transcriptional changes. In particular, an attempted reaction was activated by *P. infestans*, but it was not sufficient to contrast the negative effects of tagatose incubation on mycelial growth, sugar content and amino acid content. Therefore, *P. infestans* transcriptional adaptation was mainly manifested by the severe downregulation of genes associated with transport, sugar metabolism, signal transduction and growth-related process. On the other hand, *P. cinnamomi* response to tagatose incubation indicated the stimulation of processes related to transport, energy metabolism, sugar metabolism and oxidative stress-related, as adequate metabolic adaptation to cope with tagatose negative effects on mycelial growth, sugar content and amino acid content. Specifically, *P. cinnamomi* was able to activate multiple adaptation pathways to modulate the cellular metabolism by the upregulation of genes involved in glucose transport, pentose metabolism, tricarboxylic acid cycle, ROS detoxification, mitochondrial respiration and alternative respiration. The normal ROS level and oxygen consumption rate found in *P. cinnamomi* incubated with tagatose can be ascribed to the activation of the alternative oxidase gene. To validate the involvement of alternative oxidase gene in the mitigation of tagatose effect, *P. cinnamomi* will be incubated in the presence of tagatose and an alternative oxidase inhibitor then the ROS generation and the oxygen consumption will be measured. Similarly, the validation of the involvement of alternative oxidase in the mitigation of tagatose effect can be done by disrupting alternative oxidase gene in *P. cinnamomi* using the CRISPR/Cas approach. In addition, the

overexpression of alternative oxidase gene in *P. infestans* then evaluating the effect on mitochondrial activities is necessary to corroborate the effect of this gene in counteracting tagatose inhibitory effect.

Phytophthora infestans growth inhibition was associated with a precise reprogramming of the sugar metabolism with a decrease in glucose, glucose-1-phosphate and mannose content and β-glucosidase activity. This growth inhibition can be ascribed to structural similarities of tagatose with fructose and to competitive inhibition of key metabolic enzymes. Tagatose is the C-4 epimer of fructose and it inhibits fructose- metabolising enzymes such as fructokinase and phosphomannose isomerase of phytopathogenic oomycetes leading to glycolysis inhibition and cell wall biogenesis deficiency. However further enzymatic studies are required in order to better clarify the inhibitory effects of tagatose on *Phytophthora* spp. sugar enzymes. In addition the effect of tagatose on important phytopathogenic fungi responsible for severe economic losses can be assessed to better understand the spectrum of microorganisms inhibited by this rare sugar.

Tagatose is a promising active substance for the further development of plant protection products to control plant diseases caused by *Phytophthora* species. Although, tagatose was partially degraded by tomato phyllosphere microbiota, tagatose residues collected 1 and 7 days after plant treatment showed an inhibition of *P. infestans* growth in vitro. Further investigations are required to better understand tagatose persistence on plant grown in field. In addition tagatose affected the composition of the phyllosphere bacteria and increased the number of biocontrol agents against tomato pathogens (BCA) and the number of tomato plant growth promoting bacteria (PGPB). Therefore tagatose can have a double effect on plant health by inhibiting plant pathogens (direct effect) and by increasing the number of potential biocontrol agents which could partially contribute to disease control by competing for space, antagonism or plant resistance activation (indirect effect). Further High-throughput metagenomic analysis of plant microbial communities shift after tagatose application is required to better understand the multiple uses of this rare sugar in agriculture.

Among all tested rare sugars, only those with similar C3 spatial configuration of D-fructose (D-tagatose and L-sorbose) showed an inhibition of *P. infestans* growth, whereas those with different C3 spatial configuration (D-psicose and D-sorbose) did inhibit the growth. Thus, the structural configurations of rare sugars may be responsible for the competitive inhibition of key enzymes in sugar metabolism, and further biochemical studies are needed to verify the interactions of rare sugars with catalytic sites.

In addition, rare sugars have shown different properties in plant protection since some of them act by inducing plant resistance (e.g. D-psicose and D-allose) while others act by inhibiting the growth of phytopathogens (L-sorbose and D-tagatose). Therefore, further studies are needed to clarify whether the combination of D-tagatose with D-psicose/D-allose in crop protection strategies could complement their effects on direct plant pathogen growth inhibition and plant resistance activation.

Conclusions et perspectives

Le tagatose a inhibé la croissance de *Phytophthora infestans in vitro* et a entraîné de graves altérations mitochondriales, en particulier la formation de crêtes circulaires et concentriques. De plus, la teneur en ATP et le taux de consommation d'oxygène ont diminué, tandis que la génération des espèces réactives de l'oxygène et l'expression de gènes liés à l'apoptose et au stress oxydatif ont augmenté, ce qui suggère des déficiences graves dans les processus mitochondriaux de *P. infestans* incubé avec le tagatose. A l'inverse, la croissance et l'ultrastructure mitochondriale de *P. cinnamomi* n'ont été que légèrement affectées par le tagatose, sans effets significatifs sur la teneur en ATP et le taux de consommation d'oxygène. Les différences observées indiquent des réponses spécifiques à ce sucre rare chez deux espèces du même genre. La réponse opposée de *P. infestans* et de *P. cinnamomi* à l'incubation avec le tagatose peut être attribuée à leurs différents modes de vie qui se manifestent par des différences au niveau des hôtes (espèces de *Solanum vs* espèces annuelles et herbacées pérennes), les sites d'infection (tiges *vs* racines) et les gammes d'hôtes (étroites *vs* larges). Par conséquent, des études supplémentaires sont nécessaires pour mieux comprendre le lien entre les différents modes de vie et les différentes réponses à ce sucre rare.

La réponse différente de *P. infestans* et *P. cinnamomi* à l'incubation avec le tagatose était liée à des changements métaboliques et transcriptionnels différents entre les deux espèces. En particulier, la teneur en sucres et en acides aminés a été modifiée par le tagatose chez *P. infestans*, mais pas chez *P. cinnamomi*. De plus, suite à l'incubation avec le tagatose, un nombre élevé de gènes a été modulé chez *P. infestans* (3915 gènes) par rapport à *P. cinnamomi* (512 gènes), ce qui suggère que cette incubation a provoqué une reprogrammation transcriptionnelle sévère chez *P. infestans*, plutôt que chez *P. cinnamomi*. La reprogrammation transcriptionnelle de *P. infestans* n'aurait pas été suffisante pour contrer les effets négatifs de l'incubation du tagatose et elle a été principalement caractérisée par une sévère répression des gènes impliqués dans le transport des sucres et des acides aminés, le

métabolisme carboné, la signalisation cellulaire et les processus liés à la croissance. A l'inverse, la réponse de *P. cinnamomi* à l'incubation avec le tagatose a été caractérisée par l'activation de processus liés au transport des sucres, au métabolisme énergétique, au métabolisme carboné et au stress oxydatif pour limiter les impacts négatifs sur la croissance mycélienne, la teneur en sucre et en acides aminés. Plus précisément, *P. cinnamomi* a été capable de mettre en œuvre de multiples voies d'adaptation pour moduler le métabolisme cellulaire en se basant sur l'activation de gènes liés au transport du glucose, au métabolisme des pentoses, au cycle de l'acide tricarboxylique, à la détoxification des espèces réactives de l'oxygène, à la respiration mitochondriale et à la respiration alternative. Le niveau régulier des espèces réactives de l'oxygène et le taux de consommation d'oxygène trouvés chez *P. cinnamomi* incubé avec le tagatose peuvent être attribués à l'activation du gène de l'oxydase alternative. Pour valider l'implication de ce gène dans l'atténuation de l'effet du tagatose, *P. cinnamomi* sera incubé en présence de tagatose et d'un inhibiteur de l'oxydase alternative, puis la génération de des espèces réactives de l'oxygène et le taux de consommation d'oxygène seront mesurées. De même, la validation de l'implication de l'oxydase alternative dans l'atténuation de l'effet du tagatose peut être faite en réprimant le gène de l'oxydase alternative chez *P. cinnamomi* en utilisant l'approche CRISPR/Cas. En outre, la surexpression du gène de l'oxydase alternative chez *P. infestans* puis l'évaluation de l'effet du tagatose sur les activités mitochondriales est nécessaire pour confirmer l'effet de ce gène dans la neutralisation de l'effet inhibiteur du tagatose.

L'inhibition de la croissance de *P. infestans* causée par le tagatose était principalement basée sur l'altération des processus mitochondriaux et du métabolisme des sucres. Cette inhibition de la croissance peut être attribuée aux similitudes structurelles du tagatose avec le fructose. En fait, le tagatose est l'épimère du fructose et il inhibe les enzymes du métabolisme carboné, comme la β-glucosidase, la fructokinase et la phosphomannose isomérase des oomycètes phytopathogènes, ce qui entraîne une inhibition de la glycolyse et une déficience de la biogenèse de la paroi cellulaire. Cependant, des études d'activités enzymatiques supplémentaires sont nécessaires pour mieux comprendre les effets inhibiteurs du tagatose sur les enzymes du métabolisme carboné chez les espèces de *Phytophthora*. En outre, l'effet du tagatose contre importants champignons phytopathogènes responsables de graves pertes économiques peut être évalué afin de mieux comprendre le spectre des microorganismes inhibés par ce sucre rare.

Le tagatose est une substance active prometteuse pour le développement de produits

phytosanitaires destinés à lutter contre les maladies des plantes causées par les espèces de *Phytophthora*. Bien que le tagatose ait été partiellement dégradé par le microbiome de la phyllosphère de la tomate, les résidus de tagatose collectés 1 et 7 jours après le traitement des plantes ont montré une inhibition de la croissance de *P. infestans in vitro*. Des études supplémentaires sont nécessaires pour mieux comprendre la persistance du tagatose sur les plantes cultivées en plein champ.

En outre, les traitements avec le tagatose ont affecté la composition des bactéries de la phyllosphère et ont augmenté le nombre d'agents de biocontrôle contre les pathogènes de la tomate ainsi que le nombre de bactéries promotrices de croissance des plantes de tomate par rapport au traitement avec l'eau. Par conséquent, le tagatose peut avoir un double effet sur la santé des plantes ; en inhibant les agents pathogènes des plantes (effet direct) et en augmentant le nombre d'agents de biocontrôle qui pourraient contribuer partiellement au contrôle des maladies par compétition pour l'espace, par antagonisme ou *via* l'activation des mécanismes de défense des plantes (effet indirect). Une analyse métagénomique à haut débit des communautés microbiennes des plantes après l'application du tagatose est nécessaire pour mieux comprendre les multiples utilisations de ce sucre rare en agriculture.

Parmi tous les sucres rares testés, seuls ceux dont la configuration spatiale du carbone C3 est similaire à celle du D-fructose (D-tagatose et L-sorbose) ont montré une inhibition de la croissance de *P. infestans* alors que ceux dont la configuration spatiale du carbone C3 est différente de celle du D-fructose (D-psicose et D-sorbose) n'ont pas inhibé la croissance de *P. infestans*. Ainsi, les configurations structurelles des sucres rares peuvent être responsables de l'inhibition compétitive d'enzymes clés du métabolisme carboné et des études biochimiques supplémentaires sont nécessaires pour vérifier les interactions des sucres rares avec les sites catalytiques.

En outre, les sucres rares ont montré des propriétés différentes dans la protection des plantes puisque certains d'entre eux agissent en induisant la résistance des plantes (D-psicose et D-allose) alors que d'autres agissent en inhibant la croissance des phytopathogènes (L-sorbose et D-tagatose). Par conséquent, des études supplémentaires sont nécessaires pour montrer si la combinaison du D-tagatose avec D-psicose/D-allose dans les stratégies de protection des cultures pourrait compléter leurs effets sur l'inhibition directe de la croissance des agents phytopathogènes et l'activation de la résistance des plantes.

ANNEX

Publication 5: Interactions of tagatose with the sugar metabolism are responsible for *phytophthora infestans* growth inhibition (published in Microbiological Research)

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Interactions of tagatose with the sugar metabolism are responsible for *Phytophthora infestans* growth inhibition

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ABSTRACT

Tagatose is a rare sugar metabolised by a limited number of microorganisms that inhibits a large spectrum of phytopathogens. In particular, tagatose inhibited *Phytophthora infestans* growth and negatively affected mitochondrial processes. However, the possible effects of tagatose on *P. infestans* metabolism have not yet been investigated. The aim of this study was to analyse the impact of this rare sugar on the sugar metabolism in *P. infestans*, in order to better understand its mode of action. Tagatose inhibited the growth of *P. infestans* with a precise reprogramming of the carbohydrate metabolism that involved a decrease of glucose, glucose-1-phosphate and mannose content and β-glucosidase activity. The combination of tagatose with common sugars led to three different responses and highlighted antagonistic interactions. In particular, glucose partially attenuated the inhibitory effects of tagatose, while fructose fully impaired tagatose-mediated growth inhibition and metabolite changes. Moreover, sucrose did not attenuate tagatose effects, suggesting that the inhibition of sucrose catab- olism and the alteration of glucose-related pathways contributed to the growth inhibition caused by tagatose to *P. infestans*. The interactions of tagatose with the common sugar metabolism were found to be a key mode of action against *P. infestans* growth, which may represent the basis for the further development of tagatose as an eco-friendly fungicide.

1. Introduction

Rare sugars have been defined as monosaccharides and their derivatives that rarely exist in nature (Granström et al., 2004) and they include hexoses (e.g., tagatose, allose, gulose, and sorbose) and pentoses (e.g., lyxose, xylulose, and xylitol) (Ahmed, 2001; Jayamuthunagai et al., 2017). Conversely, common sugars are frequently found in the environment and they include well characterised sugars, such as glucose, fructose and their derivatives (Zhang et al., 2017). Although rare sugars are mainly known as low-calorie sweeteners (Levin, 2002; Matsuo et al., 2002), they acquired medical interest as anticancer (Beerens et al., 2012), anti-hyperglycaemic (Hossain et al., 2015; Lu et al., 2008), anticariogenic (Wong, 2000; Yun et al., 2017) and prebiotic molecules (Bertelsen et al., 1999; Vastenavond et al., 2012). Moreover, the development of novel enzymatic and microbial processes lowered the production costs of rare sugars and extended their application to numerous disciplines, environmental and agricultural science included (Izumori, 2006; Li et al., 2013).

Among the rare sugars, tagatose is a ketohexose, C-4 epimer of fructose and isomer of galactose, and it is used as a low-calorie sweet- ener, substitute of sucrose (Kim, 2004). Tagatose can be added to bev- erages, health foods and dietary products since it was generally recognised as safe by the Food and Drug Administration (Kim, 2004;Levin, 2002). Only about 20 % of ingested tagatose is absorbed and metabolised in the human gut (Bertelsen et al., 1999; Vastenavond et al., 2012) and the unabsorbed fraction is fermented by the gut microbiota (Bertelsen et al., 2001). However, tagatose can be used as source of carbohydrates by a restricted range of microbial taxa and it can be used only by some bacterial species as phosphorylated intermediate of the tagatose-6-phosphate pathway in the lactose, galactose and galactitol catabolism (Van der Heiden et al., 2013), indicating selective nutritional or antinutritional effects on specific microbial taxa. Tagatose is

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fermented only by specific gut microorganisms (e.g. *Enterococcus faecalis* and *Lactobacillus* spp.) (Bertelsen et al., 2001) and inhibits the growth of numerous human pathogenic bacteria (e.g. *Campylobacter* spp., *Salmonella typhi* and *Escherichia coli*) (Bertelsen et al., 2001; Lobete et al., 2017). Thus, tagatose can act as a human prebiotic product, enhancing the growth of beneficial microorganisms and inhibiting that of bacterial pathogens (Bertelsen et al., 2001; Vastenavond et al., 2012).

A similar prebiotic effect was observed in plants, where tagatose increased the relative abundance of some plant beneficial microorganisms associated with the grapevine phyllosphere (*Alternaria* spp., *Aureobasidium* spp., *Exiguobacterium* spp. and *Exophiala* spp.) (Perazzolli et al., 2020). Moreover, the main beneficial effect of tagatose ongrapevines was linked to the inhibition of oomycete and fungal phyto- pathogens, such as *Plasmopara viticola* and *Erysiphe necator* (Perazzolli et al., 2020). Tagatose can inhibit the growth of a wide range of phy- topathogens and it was patented for the control of numerous crop dis- eases, including tomato and potato late blight (Ohara et al., 2008). Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is one of the most devastating plant diseases (Agrios, 2005) and it represents a major threat to global food security, costing billions of dollars every year in terms of control efforts and production losses (Fry, 2008). As the control of late blight requires the use of chemical fungicides with po- tential harmful effects on the environment and human health (Fry, 2008), the demand for eco-friendly alternatives has increased (Axelet al., 2012). Tagatose could represent a valid alternative to chemical fungicides, thanks to the absence of negative impacts on human health (Ohara et al., 2008). However, further studies are needed on tagatose mode of action against phytopathogens and on the mechanisms under-
lying its growth inhibitory effects, in order to further develop efficient biofungicides. For instance, tagatose was able to inhibit the growth of *P. infestans in vitro* by decreasing the respiration processes and ATP content (Chahed et al., 2020), but further inhibitory effects on the metabolism of *P. infestans* have not yet been unravelled. It is known that rare sugars (e.g. psicose, sorbose and arabinose) are able to inhibit the activity of some enzymes (e.g. glucose-6-phosphate-dehydrogenase and disaccharidase) involved in the common sugar metabolism of mammalian cells (Matsuo et al., 2001; Oku et al., 2014; Seri et al., 1996). In particular, tagatose inhibited the activity of intestinal disaccharidases responsible for the sucrose and maltose metabolism (Seri et al., 1995), and one of its derivatives (tagatose-1-phosphate) was involved in the regulation of glycogen synthesis and catabolism (Agius, 1994; Lu et al., 2008). Likewise, another tagatose derivative (tagatose 6-phosphate) inhibited the transaldolase of *E. coli* (Stellmacher et al., 2016). There- fore, it is of crucial importance to investigate possible interactions be- tween the metabolism of rare and common sugars, in order to clarify the growth inhibition mechanisms. For example, tagatose caused a growth retardation of *Streptococcus mutans* and the inhibitory effect was atten- uated by the presence of fructose, but not of glucose (Hasibul et al., 2018), suggesting the presence of antagonistic interactions between rare and common sugar metabolism. However, interactions between rare and
common sugars have never been studied in the case of environmentally and agriculturally relevant microorganisms. The objective of this study was to investigate the interactions between tagatose and the metabolism of common sugars in *P. infestans*, with particular attention to the first intermediates of the glycolysis and some intermediates of the tricarboxylic acid cycle, in order to elucidate the mode of action of tagatose and to identify possible attenuation effects of common sugars on the growth inhibitory properties of tagatose.

2. Materials and methods

1. *Phytophthora infestans sugar treatment, growth conditions and radial growth assessment*

Phytophthora infestans strain VB3 was stored in glycerol at -80 °C in the fungal collection of the Fondazione Edmund Mach (Italy) and it was maintained in Petri dishes on pea agar medium (PAM, 12.5 % frozen peas and 1.2 % agar in distilled water) at 18 °C (Chahed et al., 2020).

Phytophthora infestans plugs (5 mm diameter) were cut from the edge of 10-day-old colonies and a plug was placed at the centre of each Petri dish (90 mm diameter) on PAM covered by a cellophane layer (90 mm diameter) to facilitate subsequent mycelium collection. Eight treatments were analysed, PAM as control (Control-incubated), PAM augmented with 5 g L^{-1} tagatose (Tag-incubated), 5 g L^{-1} glucose (Glu-incubated), 5 g L^{-1} fructose (Fru-incubated, 5 g L^{-1}) or 5 g L^{-1} sucrose (Suc-incubated) and PAM augmented with the combination of tagatose (5 g L^{-1}) and a common sugar (5 g L^{-1}), such as tagatose and glucose (Tag-Glu-incubated), tagatose and fructose (Tag-Fru-incubated), tagatose and sucrose (Tag-Suc-incubated). Dishes were incubated at 18 °C and *P. infestans* radial growth was assessed four and ten days after incubation (DAI), as the average of the two perpendicular diameters, subtracted the plug diameter and divided by two. Five replicates were analysed for each treatment and the experiment was carried out twice.

2. *Quantification of Phytophthora infestans metabolites*

Mycelium plugs (200 mg) were collected at 4 and 10 DAI with a sterile scalpel, frozen in liquid-N_2 and kept at 80 °C and ground using a mixer mill disruptor (MM200, Retsch, Haan, Germany) at 25 Hz for 45 s
with sterile steel jars and beads refrigerated in liquid-N_2. The metabolite extraction was carried out as previously described (Chitarrini et al., 2017) with slight modifications and the content of tagatose, glucose, fructose, galactose, mannose, sucrose, glucose-1-phosphate (gluco- se-1-P), glucose-6-phosphate (glucose-6-P), citric acid, succinic acid, malic acid, α-ketoglutaric acid, 3-hydroxy-3-methylglutaric acid (HMG), ribose and myo-inositol was measured by gas chromatography-mass spectrometry (GC–MS) analysis according to Chitarrini et al. (2017).
Briefly, 100 mg of ground mycelium was subjected to extraction by adding 900 µL of cool (20 °C) extraction solvent (iso-propanol/acetonitrile/water, 3:3:2 v/v/v), a 10 µL aliquot of a solution containing nicotinic-D4, and glucose-D7 (1000 mg L^{-1}) was added as an internal standard. The extraction mixture was vortexed for 10 s, shaken at 4 °C for 5 min and centrifuged at 12,000×g for 2 min at 5 °C. A second round of extraction was carried out following the same proced- ure. The two supernatants were merged and re-suspended in a final volume of 2 mL using the extraction solvent. A total of 200 µL of su- pernatant was placed in a 1.5 mL tube and evaporated to dryness under N_2. The residue was re-suspended in 500 mL of acetonitrile/water (50:50 v/v), vortexed for 10 s, sonicated, vortexed again 10 s and
centrifuged at 12,000 × g for 2 min. The supernatant was then transferred into a 2 mL high recovery vial and dried out under N_2. The dried extract was subjected to online derivatisation performed by the autosampler just before the injection of the sample. The derivatization procedure can be divided in two steps: first 20 µL of methoxamine hydrochloride in pyridine (20 mg mL^{-1}) was added to inhibit cyclisation of reducing sugars and shaken at 30 °C for 1 h; then 80 µL of N-meth- yl-Ntrimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane was added for the trimethylsilylation of acidic protons and shaken at 37
1. C for 30 min. Finally, 5 µL of a solution containing decane (1000 mg
L^{-1}) and heptadecane (1000 mg L^{-1}) were added in order to monitor the chromatographic analysis and the instrumental conditions. One microliter of derivatised extract was injected for GC–MS analysis. Analyses were performed using a Trace GC Ultra combined with a mass spec-trometer TSQ Quantum GC and an autosampler Triplus RSH equipped with automatic tool change (Thermo Electron Corporation, Waltham, MA, United States). An RXI-5-Sil MS w-Integra-GuardR (fused silica) (30 m 0.25 mm 0.25 mm) column was used for compound separation.
Helium was used as the carrier gas at 1.2 mL min^{-1} and the injector split ratio was set to 1:10. The injector, transfer line and source temperature were set to 250 °C. The initial oven temperature was kept at 65 °C for 2 min, increased by 5.2 °C min^{-1} to 270 °C and held at 270 °C for 4 min. The mass spectrometer was operated in electron ionisation mode. Data

acquisition was performed in full scan mode from 50 to 700 *m/z*. Data processing was performed using XCALIBUR 4.0 software (Thermo Fisher Scientific, San Jose, CA, USA).

Each metabolite was quantified as mg kg ^{1} of mycelium fresh weight, using a calibration curve of each pure compound (Sigma- Aldrich, Merc, Kenilworth, NJ, USA) dissolved in methanol:water 80:20 mix within a range of 0.01 mg L ^{1} and 1200 mg L ^{1}. Compound identification was obtained using one or, in the case of a few compounds, two specific mass to charge ratios (*m/z* ratio) characteristics for the indi- vidual metabolites (extracted ion monitoring) and excluding saturated fragments. Three replicates were analysed for each treatment. In order to obtain sufficient mycelium powder, each replicate was a pool of 35and 24 plugs in the case of Tag- and Tag-Suc-incubated samples at 4 and 10 DAI, 20 and eight plugs for the other treatments at 4 and 10 DAI, respectively.

1. 1,3-β-Glucan quantification

Mycelium plugs were collected with a sterile scalpel at 10 DAI. The content of 1,3-β-glucan (β-glucan content) was assessed using the aniline blue assay, as previously described (Fortwendel et al., 2009). Briefly, frozen mycelium of *P. infestans* was lyophilised for 24 h in a 1.5 mL-tube using LyoLab 3000 (Heto, Allerød, Denmark). The lyophilised mycelium (5 mg) was re-suspended in 500 µL of 1 M NaOH, sonicated 30 s and incubated at 52 °C for 30 min as solubilisation process and two technical replicates of each sample (50 µL) were aliquoted in a clear 96-well plate (Corning, New York, NY, USA) for β-glucan quantification and the remaining volume (100 µL) was used for protein quantification.

For β-glucan quantification, 185 µL of aniline blue mix (40 mL of 0.1 % aniline blue in sterile distilled water, 21 mL of 1 N HCl and 59 mL of 1 M glycine in NaOH buffer, pH 9.5) was added to each well. Dilutions of curdlan (Carbosynth, Berkshire, UK) were prepared as standard curve (0, 1, 5, 10, 25, 50, 100 and 200 µg mL ^{1}), from a stock solution of 1 mgmL ^{1} prepared dissolving curdlan in 1 M NaOH at 80 °C for 30 min (Shedletzky et al., 1997). The plate was incubated in the dark for 30 min at 52 °C and cooled at room temperature until fully decolorised. Fluorescence was acquired at 405 nm excitation and 460 emission using a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA).

For protein quantification, 100 µL of each sample was mixed with 100 µL of Bradford reagent (Pierce Coomassie Plus, Thermo Fisher Scientific) in a 96-well clear microplate (Corning). Samples were incubated for 10 min at room temperature and the absorbance was measured at 595 nm (A_{595}) with a Synergy 2 Multi-Mode Microplate Reader (Biotek). A standard curve of bovine serum albumin (Sigma-Aldrich; 0, 0.001, 0.005, 0.01, 0.025, 0.05 and 0.1 mg mL ^{1}) was used as reference to determine the protein concentration and the samples were diluted in sterile distilled water (appropriated dilutions to A_{595} <1.5). β-glucan content was expressed as β-glucan per unit of total proteins (mg mg ^{1}). Five replicates were used for each treatment and the experiment was carried out twice. In order to obtain sufficient mycelium powder, each replicate was a pool of 20 plugs in the case of Tag- and Tag-Suc- incubated samples and three plugs for the other treatments.

2. β-glucosidase activity assessment

Mycelium plugs (500 mg) were collected with a sterile scalpel at 10 DAI, washed twice in 50 mM phosphate buffer pH 7 and frozen at 20 °C until subsequent analysis. The frozen mycelium was ground in 1 mL of 50 mM phosphate buffer pH 7 in stainless-steel jars (pre-cooled at -80 2. C) at 20 Hz for 1 min using a mixer mill disruptor (MM200, Retsch,Haan, Germany) and centrifuged at 21,000 × g for 15 min at 4 °C. The supernatant was immediately processed using the β-glucosidase Activity

Assay Kit (Sigma-Aldrich, Merc, Kenilworth, NJ, USA), following the manufacturer instructions, and it was used for the β-glucosidase activity assay and for protein quantification.

For the β-glucosidase activity assay, two technical replicates (20 µL) of each sample were aliquoted in a clear 96-well plate (Corning) and 200 µL master mix (192.3 µL of Assay Buffer and 7.7 µL of β-NPG Substrate) were added to each sample. As blank, 220 µL of sterile distilled water was added to a well of the 96-well plate. In a different well, 20 µL of sterile distilled water was mixed with 200 µL of the calibrator providedin the kit. The initial absorbance of each sample was measured at 405nm (A_{405}) on a Synergy 2 Multi-Mode Microplate Reader (Biotek). The plate was then incubated in the dark at 37 °C for 20 min and the final A_{405} was measured. The enzymatic activity (Units mL ^{1}) was calculated according to the following formula: β-glucosidase activity = $[A_{405(final)} - A_{405(initial)}] / [A_{405(calibrator)} - A_{405(blank)}] \times 250$; where $A_{405(calibrator)}$ and $A_{405(blank)}$ were the A_{405} measured at 20 min.

For protein quantification, 100 µL of each sample was mixed with 100 µL of Bradford reagent (Pierce Coomassie Plus, Thermo Fisher Scientific) in a 96-well clear microplate (Corning). Samples were incubated for 10 min at room temperature and the A_{595} was measured with a Synergy 2 Multi-Mode Microplate Reader (Biotek). A standard curve of bovine serum albumin (Sigma-Aldrich; 0, 0.001, 0.005, 0.01, 0.025, 0.05 and 0.1 mg mL ^{1}) was used as reference to determine the protein concentration of each sample. The β-glucosidase activity was then expressed as unit of β-glucosidase per unit of total proteins (U mg ^{1}) as reported in Cordero Otero et al. (2003). Three replicates were used for each treatment and the experiment was carried out twice. In order to obtain sufficient mycelium powder, each replicate was a pool of 24 plugs in the case of Tag- and Tag-Suc-incubated samples and 10 plugs for the other treatments.

1. Statistical analysis

Data were analysed with PAST 2.17c (Hammer et al., 2001). For radial growth, β-glucosidase activity and β-glucan content the Kruskal-Wallis test was used to demonstrate non-significant differences between the two experimental repetitions ($P > 0.05$) and the data from the two experimental repetitions were pooled. For radial growth andβ-glucosidase activity, a Kruskal-Wallis test was used on untransformed data to detect significant differences among treatments ($P \leq 0.05$) based on Mann-Whitney Bonferroni corrected *P*-values ($P \leq 0.05$) (Zar, 1996). For metabolite data, the missing values were imputed with a random value between zero and the limit of quantification (LOQ) (Chitarrini et al., 2017). Data of metabolite and β-glucan content were Log_{10} transformed, the normal distribution was validated by the Shapiro-Wilk test ($P > 0.05$), variance homogeneity was assessed with the F-test ($P > 0.05$) and significant differences between sugar-incubated (Glu-, Fru-, Suc-, Tag-, Tag-Glu-, Tag-Fru- and Tag-Suc-incubated sample) and control (Control-incubated) samples were assessed for each metabolite using a parametric two-sample test (Student's *t*-test, $P \leq 0.05$) (Mar- idueña-Zavala et al., 2017). If variance homogeneity (F-test, $P \leq 0.05$) or normal distribution of the data ($P \leq 0.05$) were not satisfied, the Welch *t*-test or the Mann-Whitney test were used as non-parametric two-sampletests ($P \leq 0.05$), respectively (Xi et al., 2014). Moreover, the Kruskal-Wallis test was used to detect significant differences among treatments ($P \leq 0.05$) based on Mann-Whitney Bonferroni corrected *P*-values ($P \leq 0.05$) (Zar, 1996) on Log_{10}-transformed β-glucan content data. Changes in metabolite and β-glucan content were expressed as Log_2-transformed fold change values between sugar-incubated (Glu-, Fru-, Suc-, Tag-, Tag-Glu-, Tag-Fru- and Tag-Suc-incubated sample) and control (Control-incubated) samples, as reported by Maridueña-Zavala et al. (2017), and visualised according to pathway maps of the Kyoto Encyclopedia of Genes and Genomes (KEGG) for *P. infestans* T30 4 (T01333 KEGG). Significant differences in tagatose content between 4 and 10 DAI were assessed in Tag-, Tag-Glu-, Tag-Fru-, and Tag-Suc-incubated samples using a Student's *t*-test ($P \leq 0.05$). The Kendall non-parametric correlation analysis was run between the mean values of *P. infestans* radial growth, β-glucan content, β-glucosidase ac- tivity and metabolite content measured at 10 DAI.

3. Results

1. *Tagatose effects on Phytophthora infestans growth and metabolite content are modulated by common sugars*

Phytophthora infestans growth was lower in Tag-incubated samples compared to Control-, Glu-, Fru- and Suc-incubated samples at 4 and 10 DAI (Fig. 1). Mycelial growth in Glu-, Fru- and Suc-incubated sampleswas comparable to the growth of Control-incubated samples at 4 and 10 DAI. The combination of tagatose with a common sugar led to three different outcomes: i) radial growth in Tag-Glu-incubated samples was higher compared to that in Tag-incubated samples, but lower compared to that in Control-incubated samples at 4 and 10 DAI (partial growth inhibition of Tag-Glu incubation); ii) radial growth in Tag-Fru-incubated samples was comparable to that in Control-incubated samples at 4 DAI and higher compared to that in Tag-incubated samples at 4 and 10 DAI (restored growth of Tag-Fru incubation); iii) radial growth in Tag-Suc- incubated samples was comparable to that in Tag-incubated samples and lower compared to that in Control-incubated samples at 4 and 10 DAI (growth inhibition of Tag-Suc incubation).

In order to investigate the effects of tagatose on the common sugar metabolism, the metabolite content of *P. infestans* was assessed by GC–MS analysis. Glucose and glucose-1-P content was lower in Tag- incubated samples compared to Control-incubated samples at 4 and 10 DAI, while that of fructose and galactose was higher (Fig. 2A, Table S1). At 10 DAI, the content of sucrose and mannose was higher and lower in Tag-incubated samples compared to Control-incubated samples, respectively.

In Tag-Glu-incubated samples, glucose-1-P content was lower compared to Control-incubated samples at both time points (Fig. 2B). Glucose and galactose content was higher in Tag-Glu-incubated samples compared to Control-incubated samples at 4 DAI, while sucrose, fructose and mannose content was higher at 10 DAI (Fig. 2B). Comparing metabolite changes in Tag-Glu-and Tag-incubated samples, it was found that the presence of glucose partially attenuated metabolite changes caused by tagatose and impaired the tagatose-mediated decrease in glucose content (Fig. 2A and B).

In Tag-Fru-incubated samples, fructose and galactose content was higher compared to Control-incubated samples at both time points, mannose and sucrose content was higher at 4 DAI at 10 DAI, respectively (Fig. 2C). Overall, the presence of fructose in Tag-Fru-incubated samples (Fig. 2C) impaired the decrease in glucose, glucose-1-P and mannose content found in Tag-incubated samples (Fig. 2A).

In Tag-Suc-incubated samples, glucose and glucose-1-P content was lower compared to Control-incubated samples at 4 and 10 DAI, while fructose and galactose content was higher at both time points (Fig. 2D). Sucrose content was higher at 10 DAI in Tag-Suc-incubated samples compared to Control-incubated samples and to a greater extent compared to fold changes found in Tag-Glu- and Tag-Fru-incubated samples (Student's *t*-test, P 0.05), in agreement with the higher availability of sucrose in the media (Table S1). Metabolite changes found in Tag-Suc-incubated samples were analogous to those in Tag-incubated samples (Fig. 2A and D).

The content of glucose, glucose-1-P, glucose-6-P and galactose was comparable in Glu-, Fru-, Suc- and Control-incubated samples, with the exception of glucose and glucose-1-P in Glu-incubated samples at 4 and 10 DAI, respectively (Fig. S1). The content of one (sucrose) and two (fructose and mannose) sugars was higher in Glu-, Fru- and Suc- incubated samples compared to Control-incubated samples at one (10 DAI) and two (4 and 10 DAI) time points, respectively.

The content of succinic and malic acid was lower in Tag- and Tag- Suc-incubated samples compared to Control-incubated samples at 4 and 10 DAI, as well as that of α-ketoglutaric acid and HMG at 10 DAI (Table S1). Likewise, succinic acid content at 4 DAI and malic acid content at 4 and 10 DAI were lower in Glu- and Tag-Glu-incubated samples compared to Control-incubated samples. Myo-inositol content was higher in all sugar-incubated samples compared to Control- incubated samples at 10 DAI, except Suc- and Tag-Fru-incubated sam- ples. As expected, tagatose was detected only in Tag-, Tag-Glu-, Tag-Fru- and Tag-Suc-incubated samples, but not in Control-, Glu-, Fru- and Suc- incubated samples. Moreover, tagatose content in Tag-, Tag-Glu-, Tag- Fru- and Tag-Suc-incubated samples was comparable between 4 and 10 DAI (Student's *t*-test, $P >$ 0.05).

2. *Tagatose effects on β-glucan content and β-glucosidase activity of Phytophthora infestans are modulated by common sugars*

Phytophthora infestans β-glucan content was higher in Tag-incubated samples compared to Control-incubated samples at 10 DAI (Fig. 3). β-glucan content was higher in Glu- and Suc-incubated samples compared to Control-incubated samples, while it was comparable in Fru- and Control-incubated samples. β-glucan content was higher in Tag-Glu-, Tag-Fru- and Tag-Suc-incubated samples compared to Control-incubated samples and it was comparable to Tag-incubated samples and samples incubated with the respective common sugar (Glu-, Fru- or Suc-incubated samples).

Phytophthora infestans β-glucosidase activity was lower in Tag-incubated samples compared to Control-incubated samples (Fig. 4). In Glu-, Fru- and Suc-incubated samples, β-glucosidase activity was com- parable to Control-incubated samples. The combination of tagatose with a common sugar led to three different outcomes: i) β-glucosidase activity in Tag-Glu-incubated samples was comparable to that in Tag- and Control-incubated samples (β-glucosidase inhibition of Tag-Glu incu- bation); ii) β-glucosidase activity in Tag-Fru-incubated samples was higher compared to that in Tag-incubated samples and it was compa- rable to that in Control- and Fru-incubated samples (restored β-gluco- sidase activity of Tag-Fru incubation); iii) β-glucosidase activity in Tag- Suc-incubated samples was comparable to that in Tag-incubated

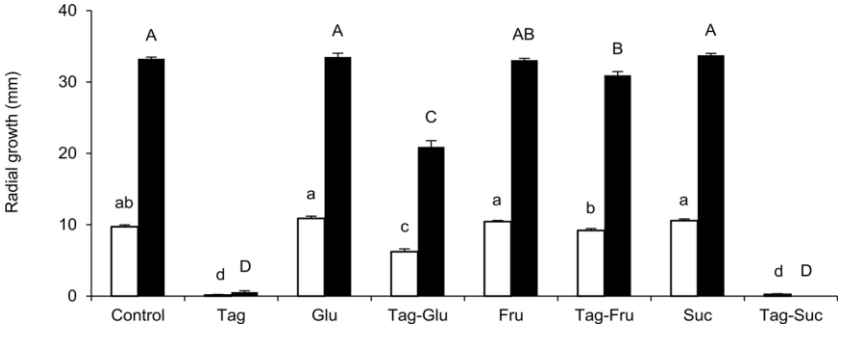

Fig. 1. Effect of tagatose and common sugars on *Phytophthora infestans* growth. *Phytophthora infestans* growth (mm) was assessed four (white) and ten (black) days after incubation (DAI) on pea agar medium in the absence (Control) and presence of 5 g L^{-1} tagatose (Tag), 5 g L^{-1} glucose (Glu), 5 g L^{-1} fructose (Fru) or 5 g L^{-1} sucrose (Suc) and in the combination of 5 g L^{-1} tagatose with 5 g L^{-1} glucose (Tag-Glu), 5 g L^{-1} fructose (Tag-Fru) or 5 g L^{-1} sucrose (Tag-Suc). The radial growth was calculated as average of the two perpendicular diameters subtracted by the plug diameter and divided by two. The Kruskal-Wallis test showed no significant differences be- tween the two experimental repetitions ($P >$ 0.05) and data from the two experiments were pooled. Bars represent mean and standard error values of ten replicates from the two experiments for each treatment. Different lowercase and uppercase letters indicate significant differences among treatments at 4 and 10 DAI respectively, according to Kruskal-Wallis test ($P \leq 0.05$).

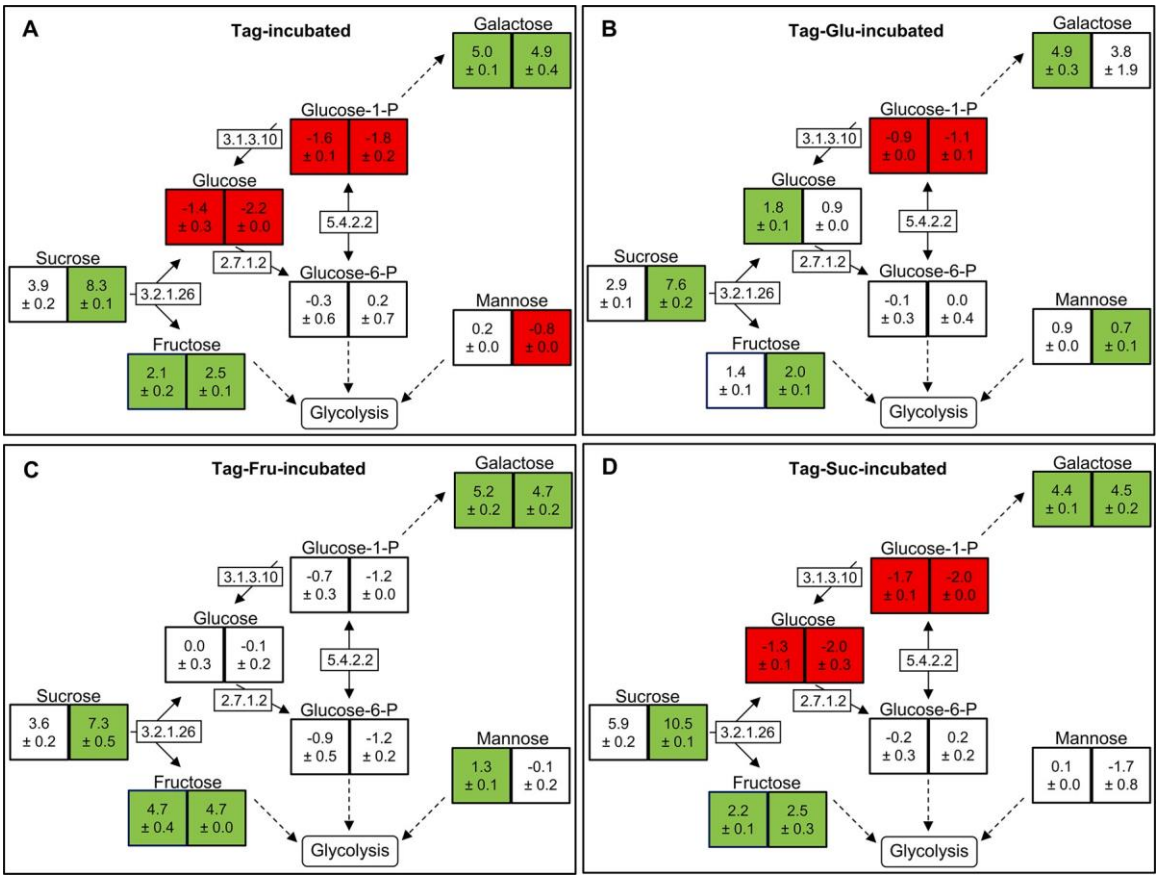

Fig. 2. Metabolite changes in *Phytophthora infestans* caused by tagatose alone and combined with common sugars. *Phytophthora infestans* metabolite content was assessed four and ten days after incubation (DAI) on pea agar medium in the absence (Control-incubated) and presence of 5 g L ^{1} tagatose (Tag-incubated), 5 g L ^{1} glucose (Glu-incubated), 5 g L ^{1} fructose (Fru-incubated) or 5 g L ^{1} sucrose (Suc-incubated) and in the combination of 5 g L ^{1} tagatose with 5 g L ^{1} glucose (Tag-Glu- incubated), 5 g L ^{1} fructose (Tag-Fru-incubated) or 5 g L ^{1} sucrose (Tag-Suc-incubated). Metabolite changes in Tag-incubated (A), Tag-Glu-incubated (B), Tag-Fru-incubated (C) and Tag-Suc-incubated (D) samples were calculated as Log_2 (fold change) values as compared with the Control-incubated samples. For each metabolite, Log_2 (fold change) mean and standard error values of three replicates at 4 DAI (left box of each metabolite) and 10 DAI (right box of each metabolite) are shown. Significantly higher (green) or lower (red) metabolite content compared to the control are highlighted, according to parametric or non-parametric two-sample tests ($P \leq 0.05$) on Log_{10}-transformed metabolite content (mg kg ^{1}). Enzyme that catalyse the key chemical reactions are reported according to the KEGG pathway maps of *P. infestans*: 3.2.1.26, invertase; 2.7.1.2, glucokinase; EC 3.1.3.10, glucose-1-phosphatase; 5.4.2.2, phosphoglucomutase. Metabolite changes caused by the common sugars are reported in Fig. S1 and the complete results of metabolite quantification are reported in Table S1. Abbreviations: Glucose-6-P, glucose-6-phosphate; Glucose-1-P, glucose-1-phosphate.

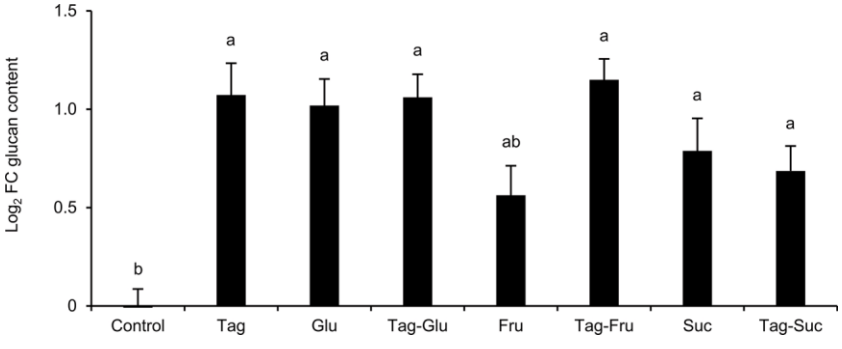

Fig. 3. Effect of tagatose and common sugars on β-glucan content of *Phytophthora infestans*. *Phytophthora infestans* β-glucan content was assessed ten days after incubation (DAI) on pea agar medium in the absence (Control) and presence of 5 g L ^{1} tagatose (Tag), 5 g L ^{1} glucose (Glu), 5 g L ^{1} fructose (Fru) or 5 g L ^{1} sucrose (Suc) and in the combination of 5 g L ^{1} tagatose with 5 g L ^{1} glucose (Tag-Glu), 5 g L ^{1} fructose (Tag- Fru) or 5 g L ^{1} sucrose (Tag-Suc). The Kruskal-Wallis test showed no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. Changes in β-glucan content were calculated as Log_2 (fold change) values between sugar- incubated (Glu-, Fru-, Suc-, Tag-, Tag-Glu-, Tag-Fru- and Tag- Suc-incubated sample) and Control-incubated samples. Bars represent mean and standard error values of ten replicates from the two experiments for each treatment. Different letters indicate significant differences among treatments according to Kruskal-Wallis ($P \leq 0.05$) on Log_{10}-transformed β-glucan content. β-glucan content, expressed per unit of total proteins (mg mg ^{1}), is reported in Table S1.

samples and it was lower compared to that in Control-incubated-samples (β-glucosidase inhibition of Tag-Suc incubation).

Overall, correlations revealed that the radial growth of *P. infestans* was positively correlated with glucose-1-P, mannose content, succinic acid, malic acid and β-glucosidase activity at 10 DAI (Table 1). β-glucosidase activity was negatively correlated with galactose content and positively correlated with ribose content, while β-glucan content was negatively correlated with glucose-1-P content, α-ketoglutaric acid,

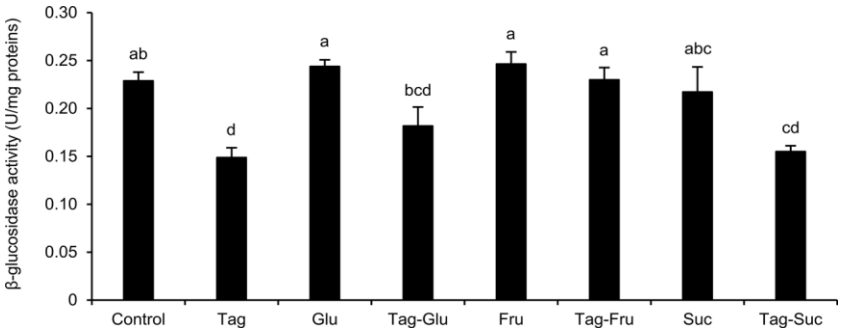

Fig. 4. Effect of tagatose and common sugars on β-glucosidase activity of *Phytophthora infestans*. *Phytophthora infestans* β-glucosidase activity was assessed ten days after incubation (DAI) on pea agar medium in the absence (Control) and presence of 5 g L^{-1} tagatose (Tag), 5 g L^{-1} glucose (Glu), 5 g L^{-1} fructose (Fru) or 5 g L^{-1} sucrose (Suc) and in the combination of 5 g L^{-1} tagatose with 5 g L^{-1} glucose (Tag-Glu), 5 g L^{-1} fructose (Tag-Fru) or 5 g L^{-1} sucrose (Tag-Suc). The Kruskal- Wallis test showed no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. The β-glucosidase activity units were expressed per unit of total proteins (U mg^{-1}). Bars represent mean and standard error values of six replicates from the two experiments for each treatment. Different letters indicate significant differences among treatments according to Kruskal-Wallis ($P \leq 0.05$).

succinic acid and HMG content.

4. Discussion

In this study, the interactions between tagatose and the metabolism of common sugars were investigated, in order to better understand its mode of action against *P. infestans*. The inhibitory effect of tagatose on *P. infestans* growth was associated with changes in the content of common sugars, such as a decrease in glucose and mannose content and an increase in sucrose, fructose and galactose content. An increase in β-glucan content and a decrease in glucose-1-P content and β-glucosi- dase activity were also observed in Tag-incubated samples, indicating the complex effects of tagatose on the sugar metabolism of *P. infestans*. In particular, the decrease in glucose and glucose-1-P content suggestedthat tagatose could increase glucose catabolism or inhibit the metabolic pathways responsible for glucose synthesis. For example, the reduced β-glucosidase activity in Tag-incubated samples possibly contributed to the decrease in glucose content, since β-glucosidases are responsible for polysaccharide (e.g. cellulose and cellobiose) (Saha et al., 1994) and disaccharide (Dhake and Patil, 2005; Saha et al., 1994) hydrolysis. The positive correlations of radial growth with β-glucosidase activity and glucose-1-P content in the different incubation conditions tested, sug-gested that glucose-related pathways are key metabolic targets oftagatose in *P. infestans*.

The combination of tagatose with common sugars led to three different *P. infestans* responses and highlighted antagonistic interactions between tagatose and common sugars in the growth inhibition pro- cesses. For example, *P. infestans* growth inhibition and metabolite changes caused by tagatose were partially attenuated by the presence of glucose. In particular, the decrease of glucose and mannose found in Tag-incubated samples was impaired in Tag-Glu-incubated samples. However, the decrease of β-glucosidase activity, glucose-1-P and the increase of β-glucan content were comparable in Tag-Glu- and Tag- incubated samples, highlighting that some tagatose effects were not affected by the presence of glucose. In Tag-Fru-incubated samples,
P. infestans growth and β-glucosidase activity were restored and the metabolite changes caused by tagatose were impaired. In particular, glucose, glucose-1-P and mannose content was comparable in Tag-Fru- and Control-incubated samples, indicating that the presence of fruc- tose impaired the effects of tagatose on *P. infestans* growth and sugar metabolism. Fructose is an epimer of tagatose and these sugars may compete for enzymatic catalytic sites through their similar chemical structures, as in the case of the mammalian fructokinase (Lu et al., 2008). Conversely, the presence of sucrose did not attenuate the effects of tagatose. In particular, *P. infestans* growth, β-glucosidase activity and changes in glucose, glucose-1-P and β-glucan content were comparablein Tag-Suc- and Tag-incubated samples. Similar interactions between rare and common sugar metabolism were also described in *S. mutans*, where the tagatose-mediated growth retardation was attenuated by the presence of fructose, but not glucose, in a growth medium containing sucrose, indicating that tagatose inhibits sucrose catabolism and that fructose mitigates tagatose effects (Hasibul et al., 2018). Similarly, when tagatose was supplied alone, or combined with sucrose, there was an inhibition of the postprandial rise in blood glucose levels in mammals (Guerrero-Wyss et al., 2018; Lu et al., 2008), suggesting that tagatose inhibited sucrose hydrolysis. Since sucrose did not restore *P. infestans* growth and sugar metabolism, tagatose possibly inhibited sucrose transport or hydrolysis, reducing the availability of energy required for *P. infestans* growth. Tagatose can inhibit the activity of disaccharidases, such as the mammalian sucrase responsible for sucrose hydrolysis (Oku et al., 2014; Seri et al., 1995). Likewise, sorbose and arabinose inhibited the sucrase activity in mammal cells (Oku et al., 2014), suggesting common inhibitory effects of rare sugars on sucrose hydrolysis. How- ever, tagatose effects were partially attenuated by the presence of glucose, suggesting that additional pathways were also affected down- stream the sucrose hydrolysis. In particular, enzymes involved in themetabolism of glucose and its derivatives were possibly inhibited by tagatose, such as the β-glucosidase activity. However, further enzymatic processes may be involved in the inhibitory properties of tagatose on
P. infestans, as found on the recombinant fructokinase and phospho- mannose isomerase of *Hyaloperonospora arabidopsidis* (Mochizuki et al., 2020). Some metabolic pathways were affected by tagatose independently of the presence of common sugars. In particular, galactose and β-glucan content increased when tagatose was supplied alone or com- bined with a common sugar. β-glucan is one of the main components of the cell wall in oomycetes (Bartnicki-Garcia and Wang, 1983) and 1,3-1, 6 β-glucan acts as intracellular storage of carbohydrates (Bulone, 2009). Tagatose possibly affected the activity of the enzymes involved in β-glucan synthesis and/or degradation, leading to an accumulation of stored polysaccharides, as found for tagatose-1-phosphate and the glycogen metabolism in mammalian cells (Agius, 1994; Lu et al., 2008). Likewise, the UDP-glucose pyrophosphorylase activity, which is impli- cated in β-glucan biosynthesis, can be increased by phosphonate treat- ment in *P. citrophthora* (Barchietto et al., 1992). In addition to sugar metabolism, the content of tricarboxylic acid cycle intermediates was affected by the presence of tagatose. In particular, malic acid, succinic acid and α-ketoglutaric acid content was lower in Tag- and Tag-Suc-incubated samples compared to Control-incubated samples, suggesting that alterations of sugar metabolism (e.g. decrease of glucose and glucose-1-P content) were accompanied by a decrease in some key organic acids for *P. infestans* growth (Rodenburg et al., 2019). In particular, malic acid and succinic acid can be metabolised as an alter- native carbon source in *P. infestans* when sugars are not available (Sa- vory et al., 2018), suggesting that their catabolism can be affected by tagatose.

The supply of a common sugar alone did not affect *P. infestans* growth, β-glucosidase activity, glucose-6-P content and, with some exception, glucose and glucose-1-P. However, an increase in sucrose, fructose and mannose content was found in Glu-, Fru- and Suc-incubated samples compared to Control-incubated samples. *Phytophthora infestans*

Table 1
Correlations among *Phytophthora infestans* radial growth, β-glucan content, β-glucosidase activity and metabolite content.

	Radial growth	β-glucan	β-glucosidase	Glucose-1-P	Glucose	Sucrose	Mannose	Galactose	Ketoglutaric acid	Succinic acid	Malic acid	HMG	Myo-inositol	Ribose
Radial growth														
β-glucan	0.21													
β-glucosidase	**0.62***	0.11												
Glucose-1-P	**0.64***	**i 0.57***	0.25											
Glucose	0.43	0.07	0.40	0.21										
Sucrose	0.57	0.21	0.33	**i 0.64***	0.29									
Mannose	**0.71***	0.07	0.55	0.50	**0.71***	0.43								
Galactose	0.56	0.48	**i 0.57***	0.48	0.24	0.40	0.40							
Ketoglutaric acid	0.50	**i 0.57***	0.18	**0.86***	0.21	0.50	0.50	0.40						
Succinic acid	**0.64***	**i 0.57***	0.40	**0.71***	0.36	0.50	0.50	**i 0.64***	**0.57***					
Malic acid	**0.64***	0.43	0.33	**0.86***	0.21	**i 0.64***	0.50	**i 0.56***	**0.86***	**0.57***				
HMG	0.54	**i 0.69***	0.24	**0.93***	0.23	0.54	0.46	**i 0.57***	**0.93***	**0.77***	**0.85***			
Myo-inositol	0.29	0.36	0.33	0.21	0.14	0.14	0.00	**0.56***	0.21	0.50	0.21	0.31		
Ribose	0.55	0.11	**0.59***	0.47	**0.62***	0.40	**0.84***	0.37	0.47	0.40	0.47	0.47	0.11	

Kendall's τ correlation coefficients are based on *Phytophthora infestans* radial growth, β-glucan content, β-glucosidase activity and metabolite content assessed at ten days after incubation on pea agar medium in the absence (Control-incubated) and presence of 5 g L^{-1} tagatose (Tag-incubated), glucose (Glu-incubated), fructose (Fru-incubated) or sucrose (Suc-incubated) or in the combination of 5 g L^{-1} tagatose with 5 g L^{-1} of glucose (Tag-Glu-incubated), fructose (Tag-Fru-incubated) or sucrose (Tag-Suc-incubated). Significant correlations are marked in bold and by asterisks ($P \leq 0.05$). Metabolite abbreviations: Glucose-1-P, glucose-1-phosphate; Ketoglutaric acid, α-ketoglutaric acid; HMG, 3-hydroxy-3-methylglutaric acid. No significant correlations were found for glucose-6-phosphate, fructose and citric acid and data were omitted.

can grow on a minimal medium augmented with a range of differentcarbon sources and it grows more efficiently in the presence of glucose, sucrose and fructose compared to mannose (Brouwer et al., 2014). Thus, *P. infestans* can modulate the metabolic pathways depending on the carbon source available and host colonisation (Rodenburg et al., 2019). For example, the addition of sucrose increased the expression levels of sucrase genes (Judelson et al., 2009) possibly to reinforce sucrose hy- drolysis and limit common sugar accumulation in the cell. The β-glucan content was higher in Glu- and Suc-incubated samples compared to Control-incubated samples, suggesting that common sugar availability increased the carbohydrate storage in *P. infestans*. Likewise, *P. cinnamomi* grown on a glucose-rich substrate accumulated β-glucans (Zevenhuizen and Bartnicki-Garcia, 1970), to cope with possible periods of starvation, as described in other microorganisms(Wilson et al., 2010).

5. Conclusions

The interactions between tagatose and the common sugar metabolism provided a better understanding of tagatose mode of action against *P. infestans* and revealed that growth inhibition was associated with a precise reprogramming of the carbohydrate metabolism with a decrease in glucose, glucose-1-phosphate and mannose content and β-glucosidase activity. The tagatose effects were partially attenuated by the presence of glucose, fully impaired by the presence of fructose and not influenced by the presence of sucrose. Thus, tagatose possibly inhibited sucrose catabolism, altered glucose-and organic acid-related pathways leading to *P. infestans* growth inhibition. Furthermore, this study provided useful information for the tagatose formulation as bio- fungicide, indicating that the presence of fructose, or other common sugars, could interfere with its efficacy. The assessment of tagatose ef- fects of on plant metabolism represents a further step in the efficacy evaluation, in order to verify possible implications on plant growth or plant resistance induction. Moreover, further transcriptomic and enzy- matic studies are required to better identify cellular processes affected by this rare sugar.

6. Author contributions

PEC conceived and carried out the experiments, data analysis, result interpretation and manuscript drafting. AN contributed to the microbiological experiments. CL carried out the metabolomic analysis. AC helped in the experimental setup. UV contributed with metabolomic data interpretation. IP conceived the study and revised the manuscript. MP conceived the study, coordinated the project and helped in manu- script drafting. All authors read and approved the final manuscript.

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Declaration of Competing Interest

AC and AN were employed by Bi-PA nv. The remaining authors declare that the research was conducted in the absence of any com- mercial or financial relationships that could be construed as a potential conflict of interest

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infestans strain VB3.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2021.126724.

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Characterization of a rare sugar against *Phytophthora* spp.

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Abstract: *Phytophthora* spp. are one of the most aggressive and widespread plant pathogens responsible for important crop losses and it is controlled by fungicides. However, the overuse of chemical fungicides is a threat to human health and the environment. Eco-friendly fungicides are needed to improve sustainable plant protection strategies. The aim of this study was to assess the efficacy and to understand the mode of action of a rare sugar (tagatose) against *Phytophthora infestans* and *Phytophthora cinnamomi*. Tagatose inhibited *P. infestans* growth, but not *P. cinnamomi* growth, and it altered respiration-related activities. We demonstrated that tagatose is a promising compound that could be used as innovative biopesticide for the control of *P. infestans*.

Key words: rare sugar, *Phytophthora* spp., biological control, antioomycete

Introduction

Rare sugars are defined as monosaccharides and their derivatives that rarely exist in nature (Granström et al., 2004). Among them tagatose inhibited the growth of phytopathogens and it was patented for the control of important crop diseases, such as tomato and potato late blight (*Phytophthora infestans*) (Ohara et al., 2010). Tagatose could be a promising alternative to synthetic chemical fungicides, thanks to the absence of deleterious effect for human health (Levin, 2002; Vastenavond et al., 2011). However, the mode of action of tagatose on plant-associated microorganisms is unknown. The aim of this study was to elucidate the mode of action of tagatose in two phytopathogenic *Phytophthora* spp. *in vitro* and to provide deeper knowledge for the further development of eco-friendly fungicides.

Material and methods

Assessment of tagatose impact on Phytophthora spp. growth

Phytophthora infestans and *P. cinnamomi* were grown in Petri dishes on pea agar medium (PAM, 12.5% frozen peas and 1.2% agar in distilled water) at 18 °C and 25 °C, respectively (Puopolo et al., 2014). The radial growth of *P. infestans* and *P. cinnamomi* was assessed four and ten days after incubation (DAI) at 18 °C and 25 °C, respectively, as average of the two perpendicular diameters subtracted by the plug diameter and divided by two.

Assessment of the oxygen consumption rate and reactive oxygen species accumulation
The *P. infestans* and *P. cinnamomi* mycelial suspension was incubated in the absence (control) and presence of 5 g/l tagatose in pea broth in 96-well microplate under orbital shaking at 80 rpm at 18 °C and 25 °C, respectively. The oxygen consumption rate (OCR) was measured using the MitoXpress Xtra Oxygen Consumption Assay (Luxcel Biosciences) and Intracellular ROS were quantified with H_2DCF-DA (Molecular Prob according to the manufacturer s instructions.
,

Results and discussion

Phytophthora infestans growth was inhibited by tagatose at 4 and 10 DAI and the inhibitory effect was comparable between 5 g/l and 10 g/l tagatose (Figure 1 A). Conversely, *P. cinnamomi* growth was slightly inhibited by only 10 g/l tagatose at 4 DAI (Figure 1 B), indicating that tagatose exhibited differential activities against species belonging the same genus.

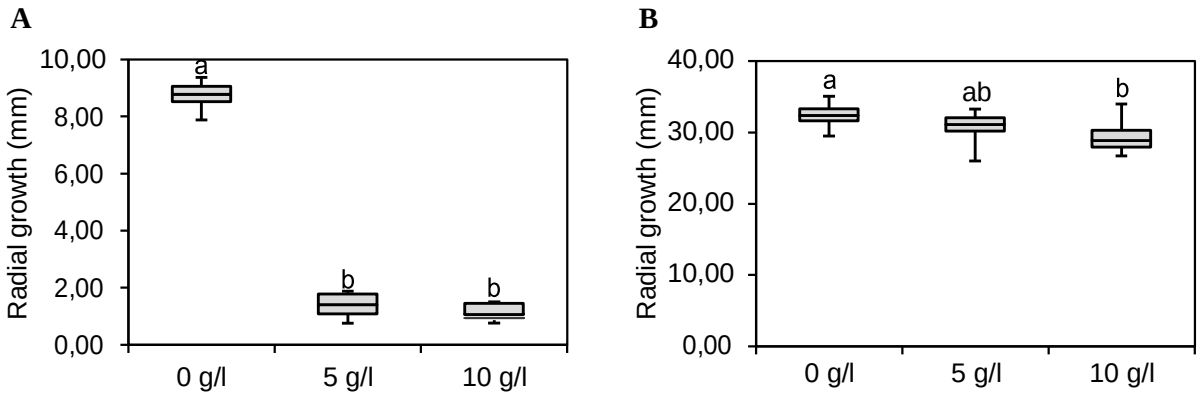
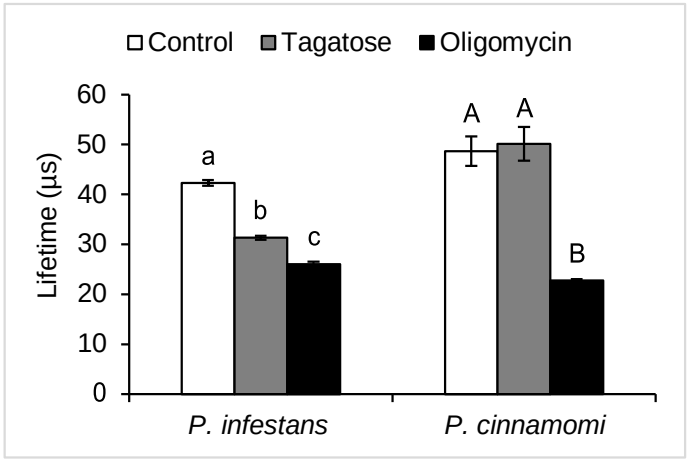


Figure 1. Effect of tagatose on *Phytophthora* spp. growth. *Phytophthora infestans* (A) and *P. cinnamomi* (B) growth (mm) was assessed four days after incubation (DAI) on pea agar medium in the absence (0 g/l) and presence of 5 g/l or 10 g/l tagatose. Different letters indicate significant differences according to the Kruskal-Wallis's test ($P \leq 0.05$).

The OCR was inhibited and ROS production was increased in tagatose-incubated *P. infestans* (Figure 2 A), suggesting inhibition of mitochondrial processes. On the other hand, the OCR and ROS generation were not affected by tagatose in *P. cinnamomi* (Figure 2 B).

Tagatose inhibited *P. infestans* growth and caused a reduction in the OCR and an increase of the ROS level, indicating severe deficiencies in tagatose-incubated *P. infestans*. On the other hand, *P. cinnamomi* growth, OCR and ROS accumulation were not affected by tagatose, indicating species-specific responses to this rare sugar. Further studies are required to better understand tagatose impacts on mitochondrial processes and gene expression regulations in *P. infestans* and *P. cinnamomi*.

A



B

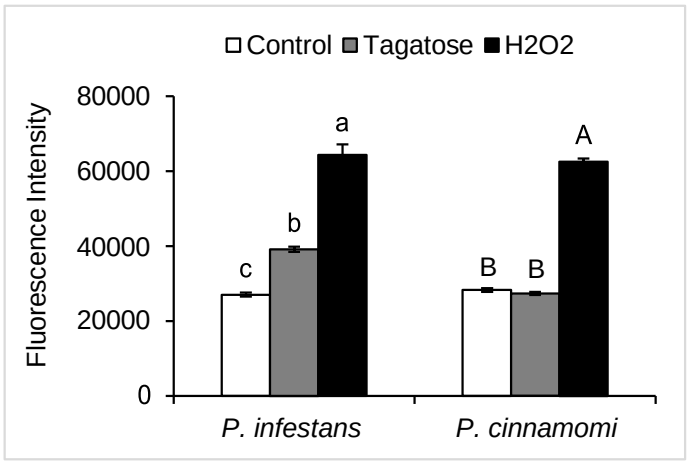


Figure 2. Effect of tagatose on *Phytophthora* spp. respiration processes and accumulation of reactive oxygen species**.** The oxygen consumption rate (A) and the generation of reactive oxygen species (B) of *P. infestans* and *P. cinnamomi* mycelial suspension was assessed 16 h after incubation in pea broth in the absence (white) and presence of 5 g/l tagatose (grey). Oligomycin and hydrogen peroxide (H_2O_2) were used as control treatment of the OCR and ROS assay (black), respectively. Different letters indicate significant differences according to the Kruskal-Wallis's test ($P \leq 0.05$).

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Dissemination:

Publications:

1/ The rare sugar tagatose differentially inhibits the growth of *Phytophthora infestans* and *Phytophthora cinnamomi* by interfering with mitochondrial processes (doi: 10.3389/fmicb.2020.00128)

2/ Interactions of tagatose with the sugar metabolism are responsible for *Phytophthora infestans* growth inhibition (doi: 10.1016/j.micres.2021.126724)

3/ Characterization of a rare sugar against *Phytophthora* spp. IOBC-WPRS Bulletin (2020) 153: 1-4

4/ A review of knowledge on the mechanisms of action of the rare sugar D- tagatose against phytopathogenic oomycetes (submitted to Plant Pathology)

5/ The differential growth inhibition of *Phytophthora* spp. caused by the rare sugar tagatose is associated with species-specific metabolic and transcriptional changes (submitted to mSystems)

6/ Persistence of the rare sugar tagatose on tomato leaves and its effect on the phyllosphere bacteria (future submission to Microorganisms)

Presentations:

1/ Oral presentation ‘’Characterization of an innovative natural molecule against plant pathogens’’ during the Trial Field Days organized by Belchim Crop Protection, Londerzeel - Belgium (2018, June)

2/ Three minutes Flash talk “Characterization of the anti-oomycete activity of a sugar against *Phytophthora* spp.” at the Midlands Molecular Microbiology Conference, Nottingham - UK (2019, September)

3/ Oral presentation “A sugar derivative inhibits the growth of *Phytophthora infestans* and not *Phytophthora cinnamomi* by impairing respiration-related processes” within the IOBC-WPRS working group Integrated Protection of Field Vegetables, Stratford Upon Avon - UK (2019, October)