RESEARCH ARTICLE

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In vitro and *in vivo* antifungal activity of plant extracts against common phytopathogenic fungi

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ABSTRACT
Natural crop protection products are gaining popularity due to consumer demands and the long-term toxicity of chemical pesticides. Extracts of nine locally available plant species were screened for their fungicidal activity against four widespread fungal phytopathogens. Examination of the effect of the plant extracts on mycelial growth was done *in vitro* using a liquid culture medium. During this assay, all tested plant extracts showed significant antifungal activity ranging from 24% up to 60% decrease in mycelial growth compared to the blank treatment. Extracts of *Fallopia japonica* and *Artemisia vulgaris* caused the greatest decrease in mycelial growth. Four plant extracts were used for an *in vivo* assay on cherry tomatoes. All selected plant extracts managed to limit the fungal growth of a phytopathogen *in vivo*. However, no extract managed to visually inhibit the growth of all four fungal strains. The *in vitro* results propose plant extracts be a promising alternative to hazardous chemical fungicides. While the *in vivo* assay was less persuasive, an effective match between a plant extract and a single phytopathogen appears to be achievable in the near future.

Key words: fungicidal activity, plant extracts, *in vitro* assay, *in vivo* assay, plant pathogens, crop protection products

Introduction

Legislation concerning the use of crop protection products (CPPs) for agricultural and private applications is getting more strict. Key features of newly approved CPPs are biodegradability, long-term toxicity to the environment, specificity of the product for target organisms, and health effects on the applicator ("EUR-Lex – 32012R0528"). The demand for CPPs of natural origin is increasing since these tend to be more environmentally friendly. Additionally, they generally have a higher specificity to the pest that must be controlled compared to broad-spectrum CPPs (Zaker, 2016). Furthermore, biopesticides meet the growing consumer demand for low or no residues on agricultural crops. Consumers increasingly ask for natural products, also driving the expansion of biopesticides to the private garden segment (Marrone, 2014). A safety evaluation of several botanical pesticides conducted by Hernández-Moreno et al. (2013) stated that the use of clove and pyrethrum is no safety concern for application by farmers or consumers.

Before an active substance can be used within a CPP in the European Union it must be approved by the European Commission. Substances undergo an intensive evaluation and peer-review by the Member States and the European Food Safety Authority before a decision on approval can be made. This is a costly procedure. Therefore, companies are keen to know whether a naturally sourced CPP can be economically profitable and has high efficiency in fighting plant pathogens, before starting this approval procedure.

This study focuses on the effects of extracts from local plants on the control of some fungi which are causing agricultural losses globally. For example, *Botrytis cinerea* causes gray mold disease and is the second-most harmful fungal phytopathogen. It affects more than 586 genera and 1400 species of crops (Yang et al., 2020). As a result of its broad host range, it can decrease, 20 to 30% of crop production annually. Fungicides that specifically targeted *Botrytis* ("botryticides") cost €540 million in 2001, representing 10% of the world fungicide market (Dean et al., 2012). Another fungus ranked in the "Top 10" of fungal plant pathogens is *Fusarium oxysporum* (Dean et al., 2012). It causes vascular wilt and root rot on a wide range of plants. This fungal plant pathogen can infect more than 100 different hosts, thanks to its different formae speciales (ff. spp.). However, these individual strains display selective pathogenicity to a narrow range of host plants (Edel-Hermann & Lecomte, 2019). *Rhizoctonia solani* is a soil-borne pathogen, like *F. oxysporum*. It has a wide range of hosts as well, however, it is mostly known for causing black

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scurf on potato tubers. Losses can go up to 30% due to the disease (Bokhari et al., 2015). Rice is another economically important crop affected by *R. solani* (Ali et al., 2017). Last but not least, *Sclerotinia minor* causes devastating diseases in at least 94 species, including lettuce, peanuts, and potatoes (Melzer et al., 1997; Hollowell et al., 2003; Matheron & Porchas, 2019). However, *S. minor* also serves a positive use: it is also an active ingredient in SARRITOR™, registered in Canada as a bioherbicide against dandelions (Hershenhorn et al., 2016).

For CPPs of plant origin, the availability of plant parts out of which the active substance can be extracted is an important factor. For this research, the aim was to investigate which locally sourced plants and agricultural waste streams are contenders for becoming an economically and ecologically profitable CPP. The focus was to use green solvents and sustainable extraction techniques. Several extraction procedures for plant extractions have already been described in the scientific literature (Park et al., 2008; Nikolova et al., 2017; Wavare et al., 2017). However, most are "overkill"-procedures with great solvent-substrate ratios and extended extraction periods. This study uses Pressurized Liquid Extraction (PLE) to produce plant extracts. Compared to traditional solvent-liquid extractions at atmospheric pressure, PLE enhances extraction performance (Mustafa & Turner, 2011). The extraction solvent of choice is a mixture of ethanol and water since simple alcohols are more environmentally friendly compared to many other organic solvents. Furthermore, alcohol-water mixtures are preferred from an environmental point of view compared to pure alcohol (Capello et al., 2007). Optimization of the extraction procedures greatly enhances the economic viability of the plant extracts as potential CPPs. The production of an effective crude and unrefined plant extract with high antifungal activity against the target organisms is the ideal outcome. Using crude extract rules out the necessity of isolating the active substances, which avoids an energy-intensive industrial process and the generation of a big waste stream.

Materials and Methods

Fungal pathogens and plant materials

Isolates of fungal strains were kindly donated by Belchim (Londerzeel, Belgium) and Bi-Pa (Londerzeel, Belgium). The fungal strains were *Botrytis cinerea* isolated from lettuce, *Fusarium oxysporum* f. sp. radicis-lycopersici, *Rhizoctonia solani* AG2-1, and *Sclerotinia minor*.

Raw plant materials were kindly supplied by experimental farm Rusthoeve AIKC (Colijnsplaat, The Netherlands). The vegetal material used in this study is listed in Table 1. Plant materials were selected based on the ability to be grown locally easily in The Netherlands and Belgium, combined with literature research. All plant materials were washed to remove dirt and debris and air-dried before being stored at -20 °C until further use.

Production of plant extracts using Accelerated Solvent Extraction (ASE)

Plant materials were freeze-dried using a lyophilisator (Alpha 1-2 LDplus) and ground in a kitchen chopper (Moulinex DP810855). The dry and ground plant material was extracted via accelerated solvent extraction using a Dionex ASE 350® apparatus. One gram of plant material was extracted with approximately 45 mL of 70:30 ethanol:water. The solvent was added automatically to build pressure in the extraction cell. The plant material was introduced into a 5 mL ASE cell together with 0.5 g Celite® 545 diatomaceous earth. The cells were equipped with a cellulose filter (Thermo Scientific) at the bottom to avoid the collection of plant debris in the collection vial. Diatomaceous earth was used to reduce the solvent volume and avoid dense cake formation inside the ASE cell. Three extraction cycles with the following parameters were performed: the cell containing the sample was filled with the extraction solvent, pressurized (1500 psi), and then heated at 70 °C for 5 min followed by a static period of 5 min. Then, the cell was rinsed with fresh extraction solvent (100% of the extraction cell volume), pressurized, heated, and underwent another 5 min static period. After this static period, the cell was purged with nitrogen flow. Extracts were collected into 60 mL glass vials. Once the extraction was finished, the ethanol fraction was

Table 1: *Plant materials tested for the antifungal activity of its extracts.*

Scientific name	Common name	Plant parts used
Anacyclus pyrethrum	Spanish chamomile	Aerial parts
Artemisia vulgaris	Common mugwort	Aerial parts
Chrysanthemum coccineum	Pyrethrum	Flowers
Chrysanthemum coccineum	Pyrethrum	Aerial parts
Fallopia japonica	Japanese knotweed	Aerial parts
Sonchus arvensis	Field milk thistle	Aerial parts
Tanacetum vulgare	Tansy	Aerial parts
Taraxacum kok-saghyz	Russian dandelion	Whole plant
Taraxacum officinale	Common dandelion	Whole plant

removed using a Büchi R-210 rotary evaporator (60 °C, 200 mbar). This is necessary since ethanol itself has an antifungal effect (Kurita & Koike, 1983). The leftover aquatic fraction was centrifuged in sterile centrifuge tubes for 5 min at 4500 rpm (Sigma 3-16PK). The supernatant was refrigerated until further use. A reference blank solution was produced in the same way. In the case of the production of the blank, the ASE cell was filled with Celite® 545 diatomaceous earth alone. Afterward ethanol was removed from the extraction solvent using a rotary evaporator, leaving only the aquatic fraction.

Homogenized inoculum production

The inoculum was prepared by culturing the fungal species on potato dextrose agar (PDA) in darkness at 25 °C for 7 days in 9 cm Petri dishes. The preparation of homogenized mycelium for inoculation of liquid culture flasks was based on the method described by Zweck et al. (1978). In a 50 mL conical flask with a screw cap, 25 mL of potato dextrose broth (PDB) was introduced and sterilized (15 min, 121 °C). Agar plugs were removed from the culture plates using a sterile 6 mm cork borer. A sterilized scalpel was used to transfer the agar plugs into the liquid PDB medium. Depending on the growth rate of the fungal species, the amount of agar plugs varied; *B. cinerea*: 3 plugs, *F. oxysporum*: 3 plugs, *R. solani*: 4 plugs, *S. minor*: 5 plugs. With the screw cap slightly opened, the conical flasks were placed on a shaking incubator at 25 °C and 180 rpm (VWR incubating mini shaker). Once the medium was turbid (± 72 h of incubation), the culture medium was transferred to sterile centrifuge tubes while the agar plugs were left out. The medium was centrifuged for 5 min at 3893 x g and the supernatant was discarded. The mycelium left in the centrifuge tube as a pellet was lyophilized and refrigerated until further use. Using a sterile glass rod, the dried mycelium was ground. The ground mycelium was dissolved in sterile distilled water at 2 g/L to produce inoculum for the antifungal assays. If flakes were visible in the solution, it was placed in an ultrasound bath (VWR USC 600 TH). Prepared inocula were refrigerated until used for further experiments. All steps were performed in a laminar air flow cabinet (FasterAir UCS 2-4) to prevent contamination of the fungal cultures.

In vitro antifungal assay

A gravimetric method adapted from Guha et al. (2005) was used to assess the efficacy of the unrefined aquatic plant extracts against the target fungal strain growing in a liquid PDB medium. For the *in vitro* antifungal assay, 25 mL of fresh PDB was introduced into a 50 mL conical flask with a screwcap and sterilized (15 min, 121 °C). Afterward, either 500 µL of a blank solution or the supernatant of the aquatic plant extract was added. At the same time, 200 µL of the prepared inoculum was added. With the screw cap slightly opened, the conical flasks were placed on a shaking incubator at 25 °C and 180 rpm. After 72 h of incubation, the culture medium was transferred to pre-weighed centrifuge tubes and centrifuged for 5 min at 3893 x g. The supernatant was discarded. The mycelium left in the centrifuge tube as a pellet was lyophilized and weighed. A 500 µL aliquot of the supernatant of the aquatic plant extract was lyophilized as well to be able to correct for the dry residue of the plant extract. All steps were performed in a laminar airflow cabinet to prevent contamination of the fungal cultures. The antifungal capacity of each plant extract was assessed on each fungal species and mean values were considered with standard deviations (SD). The efficacy of the plant extract was expressed in percentages relative to fungal cultures treated with the blank solution and calculated using Eq. 1-3:

$$\text{Dry fungal biomass (g/25 mL) } m_{b1} = m_{b+c+t} - m_c - m_t \quad (1)$$

$$\text{Dry fungal biomass (mg/mL) } m_{b2} = \frac{m_{b1}}{25 \times 1000} \quad (2)$$

$$\text{Efficacy compared to blank (\%)} = \frac{m_{b2p} - m_{b2b}}{m_{b2b}} \times 100 \quad (3)$$

where,

m_{b+c+t} is the mass of the centrifuge tube with the lyophilized pellet containing the fungal mycelium and dry residue of the blank solution or supernatant of the aquatic plant extract,

m_c – the mass of the empty centrifuge tube,

m_t – the mass of the dry residue of a lyophilized 500 µL aliquot of the blank solution or supernatant of the aquatic plant extract,

m_{b1} – the dry fungal biomass expressed in g/25mL,

m_{b2} – the dry fungal biomass expressed in mg/mL,

m_{b2p} – the dry fungal biomass expressed in mg/mL with plant extract treatment,

m_{b2b} – the dry fungal biomass expressed in mg/mL with blank solution treatment.

In vivo antifungal assay

For the *in vivo* assays, tomatoes were used to assess the protective capacity of the plant extracts against different fungal species based on Yang et al. (2020). Fresh and healthy cherry tomatoes (*Solanum lycopersicum*) were disinfected with 70% ethanol and rinsed with sterile distilled water. The fruits were left to air dry. A thin layer of the fruit's skin was cut off, leaving 1 cm² of the inside exposed. Afterward, the fruits were immersed in a beaker of the blank solution or aquatic plant extract for 5 min and left to air dry. Three cherry tomatoes were used for each treatment or for the blank. Agar plugs were removed from the culture plates using a sterile 3 mm cork borer. The plugs were taken from the edge of the circular growing zone of the fungus. One agar

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plug was placed on the exposed area of each fruit, with the mycelium face down on the surface. To promote infection, treated fruits were kept in sterilized plastic containers on a layer of moistened paper tissues. The plastic containers were incubated at 25 °C in darkness (MMM Venticell). The progress of the rotting area caused by the fungal species was visually assessed every day for seven days post-inoculation.

Statistical analysis

Outliers of replicate data were identified using the interquartile range and left out for further calculations (Supplemental data, Table S1-S4). The Student's t-test was applied to determine significant differences between means of treated and blank samples. Analysis of variance (ANOVA) was used to evaluate significant differences in percentage inhibition caused by the plant extracts.

Results

Evaluation of in vitro antifungal activity of plant extracts

The plant extracts' *in vitro* antifungal activity was evaluated by determining the inhibition of mycelial growth of each fungal species under study. Flasks were incubated at 25 °C with shaking at 180 rpm for 72 h. The biomass produced in each culture flask was removed by centrifugation and freeze-dried and the average dry biomass was determined (Supplemental data, table S1-S4). The percentage of growth inhibition was calculated by comparing the average dry biomass with plant extract added relative to the average dry biomass with blank solution added. The growth inhibition of each fungal species caused by the plant extracts is shown in Figures 1-4.

Overall, all plant extracts have a significant inhibiting effect on the mycelial growth of all fungal species under study. The only exception is Russian dandelion extract (*T. kok-saghyz*), which does not succeed in significantly inhibiting the growth of *B. cinerea* and *R. solani* compared to the blank treatment (Figure 3-4).

The antifungal effects of all plant extracts are not of the same extent. Japanese knotweed (*F. japonica*) and common mugwort (*A. vulgaris*) cause the greatest effects, with an average inhibition of 60% and 51% respectively. All other plant extracts do not differ significantly according to the ANOVA test ($p = 0.71$) and cause mycelial growth inhibitions of 24 to 34%. The growth of all four fungal species under study was inhibited by the addition of the plant extracts. The extent of inhibition is not significantly different between fungal species according to the ANOVA test ($p = 0.09$). The percentage inhibition values are presented in Table 2.

Evaluation of in vivo antifungal activity of plant extracts

The plant extracts' *in vivo* antifungal activity was evaluated by placing an agar plug colonized by each fungal species on the cut-off surface of cherry tomatoes. Beforehand the fruits were immersed in a beaker of the blank solution or aquatic plant extract for 5 min. After inoculation, the progress of the fungal growth caused by the fungal species was visually assessed daily, as shown in the example in Figure 5. The results of the *in vivo* assay are summarized in Table 3.

R. solani and *B. cinerea* each showed a decreased fungal growth caused by three different plant extracts. *F. oxysporum* growth was only decreased due to the treatment with *A. vulgaris* extract. At last, *S. minor* growth was not affected by either one of the plant extracts tested during the *in vivo* assay.

Table 2. *Percentage inhibition of mycelial growth of fungal species under study caused by the addition of 500 µL of plant extract compared to the blank sample. Means ± SD labeled with different letters were significantly different according to ANOVA test at $p < 0.05$.*

Plant extract	***F. oxysporum***	***B. cinerea***	***R. solani***	***S. minor***	**Mean percentage inhibition per plant extract**
A. pyrethrum	26%	19%	26%	24%	24% ± 3% [a]
A. vulgaris	37%	22%	72%	71%	51% ± 25% [b]
C. coccineum	27%	22%	33%	27%	27% ± 5% [a]
C. coccineum flowers	24%	24%	20%	36%	26% ± 7% [a]
F. japonica	43%	55%	72%	69%	60% ± 13% [b]
S. arvensis	31%	25%	51%	28%	34% ± 12% [a]
T. vulgare	32%	24%	23%	28%	27% ± 4% [a]
T. kok-saghyz	22%	9%	17%	47%	24% ± 16% [a]
T. officinale	20%	24%	31%	51%	31% ± 13% [a]
Mean percentage inhibition per fungus	29% ± 8% [c]	25% ± 12% [c]	38% ± 21% [c]	42% ± 18% [c]	

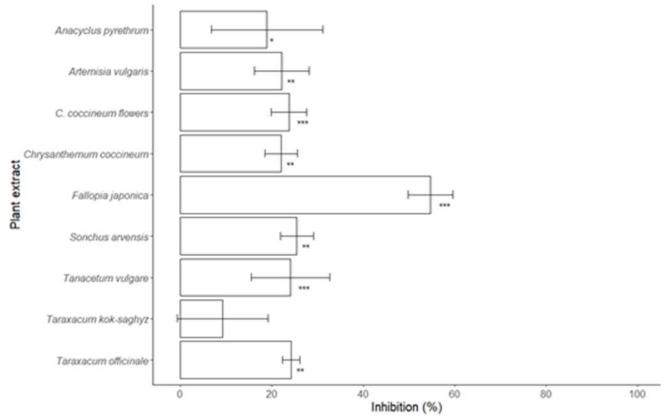

Figure 1. *Percentage inhibition of mycelial growth of Botrytis cinerea caused by the addition of 500 µL of plant extract compared to the blank sample. Error bars indicate standard deviations of replicates. Bars labeled with asterisks were significantly different compared to the blank treatment according to student's t-test. * = p < 0.05; ** = p < 0.01; *** = p < 0.001.*

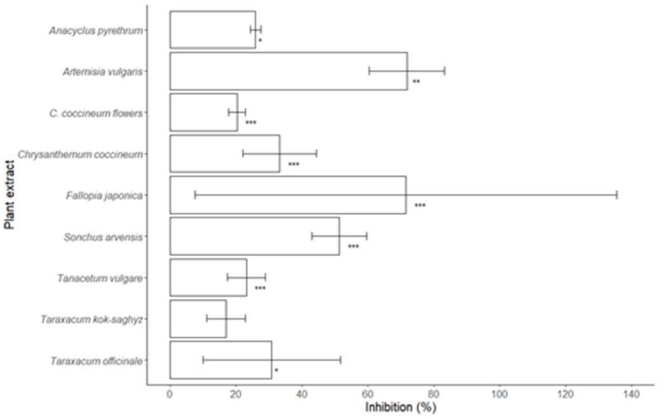

Figure 3. *Percentage inhibition of mycelial growth of Rhizoctonia solani caused by the addition of 500 µL of plant extract compared to the blank sample. Error bars indicate standard deviations of replicates. Bars labeled with asterisks were significantly different compared to the blank treatment according to student's t-test. * = p < 0.05; ** = p < 0.01; *** = p < 0.001.*

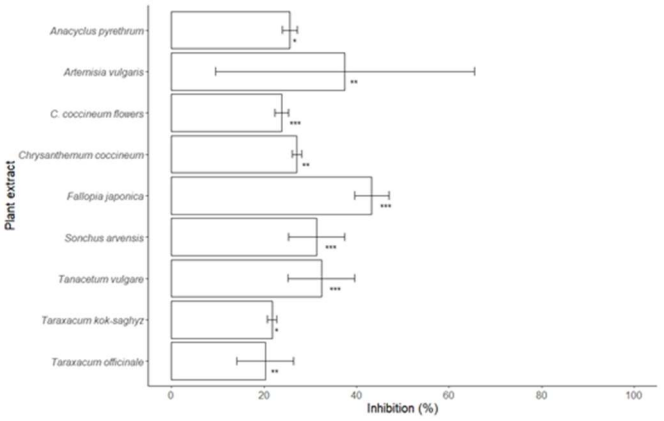

Figure 2. *Percentage inhibition of mycelial growth of Fusarium oxysporum caused by the addition of 500 µL of plant extract compared to the blank sample. Error bars indicate standard deviations of replicates. Bars labeled with asterisks were significantly different compared to the blank treatment according to student's t-test. * = p < 0.05; ** = p < 0.01; *** = p < 0.001.*

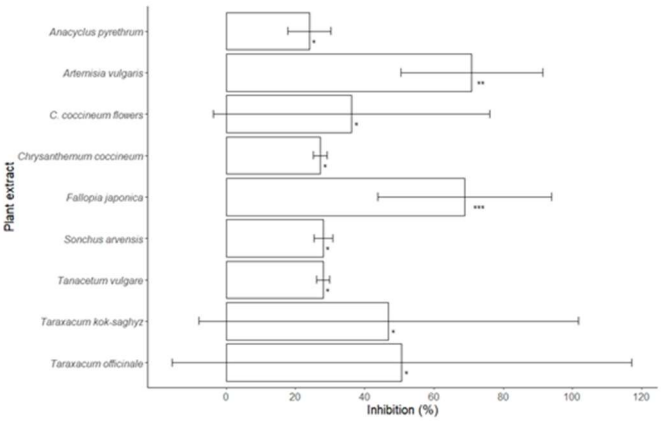

Figure 4. *Percentage inhibition of mycelial growth of Sclerotinia minor caused by the addition of 500 µL of plant extract compared to the blank sample. Error bars indicate standard deviations of replicates. Bars labeled with asterisks were significantly different compared to the blank treatment according to student's t-test. * = p < 0.05; ** = p < 0.01; *** = p < 0.001.*

Table 3. *Summary of the visually assessed fungal growth per plant extract treatment compared to treatment with blank solution during the in vivo assay.*

Plant extract	***B. cinerea***	***F. oxysporum***	***R. solani***	***S. minor***
Sonchus arvensis	decrease	no effect	no effect	no effect
Tanacetum vulgare	decrease	no effect	decrease	no effect
Fallopia japonica	decrease	no effect	decrease	no effect
Artemisia vulgaris	no effect	decrease	decrease	no effect

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Discussion

The use of chemical fungicides to control phytopathogens has many drawbacks and can be hazardous to the environment and applicator. Many weeds and agricultural waste streams are nowadays considered to be useless or bothersome. In this study, the antifungal potential of locally available weeds was investigated using an *in vitro* and *in vivo* assay. The use of plant extracts as antifungal substance phytopathogens has been reported before (Park et al., 2008; Al-Rahmah et al., 2013; Singh, 2014). The *in vitro* assay showed that all assessed plant extracts demonstrated significant inhibition of fungal biomass production compared to a blank solution. However, this inhibition varied greatly between plant species. The smallest average inhibition of -24% was caused by extracts of *A. pyrethrum* and *T. kok-saghyz*, while the greatest average inhibition of -60% and -51% was linked to the addition of *F. japonica* and *A. vulgaris* extract respectively to the liquid growth medium of four common phytopathogens. Nikolova et al. (2017) reported inhibition effects of aqueous-methanolic extracts of *Artemisia annua* on mycelial growth of phytopathogens *Alternaria alternata* and *Botrytis cinerea*. The efficacy of *F. japonica* extracts is promising and presents an opportunity to valorize Japanese knotweed cutoffs. In Belgium and the Netherlands, it is an invasive alien plant which is extremely difficult to control by both manual and chemical methods (Tiébré et al., 2008). Finding a useful purpose for the manually removed plant parts can give a positive twist to the unsolicited success of this invasive plant species. Anžlovar et al. (2020) however reported growth stimulation of some fungal species caused by extracts of *F. japonica* leaves and rhizomes. While most error bars in Figures 1-4 are acceptable, the large error bars in Figure 4 may indicate the used method was not adequate for monitoring the growth of *Sclerotinia minor*. However, the trend caused by the addition of plant extracts is still apparent.

In contrast to the *in vitro* results, which show great potential for the possibility to use the tested plant extracts as natural fungicides, the *in vivo* results were less persuasive. No plant extract succeeded in inhibiting the fungal growth of all fungal species under investigation. *T. vulgare* and *F. japonica* extracts, each visually inhibited the growth of two fungal species. *R. solani* and *B. cinerea* showed a decreased fungal growth caused by the treatment of these plant extracts. *F. oxysporum* was only visually inhibited by the *A. vulgaris* extract. At last, *S. minor* growth was not visually inhibited by any of the plant extracts included in the *in vivo* assay, while *A. vulgaris* extract caused an average -51% decrease in mycelial growth during the *in vitro* assay. This discrepancy between the results of *in vitro* and *in vivo* antifungal assays was also present during the work of Muñoz Castellanos et al. (2020), where some essential oils were tested for their activity against *F. oxysporum* in tomatoes.

In conclusion, plant extracts show to be a promising alternative to hazardous synthetic fungicides. However, finding a single plant extract working effectively against a broad range of phytopathogenic fungi will be a challenging task. An effective match between a plant extract and a single fungus is an achievable goal in the near future.

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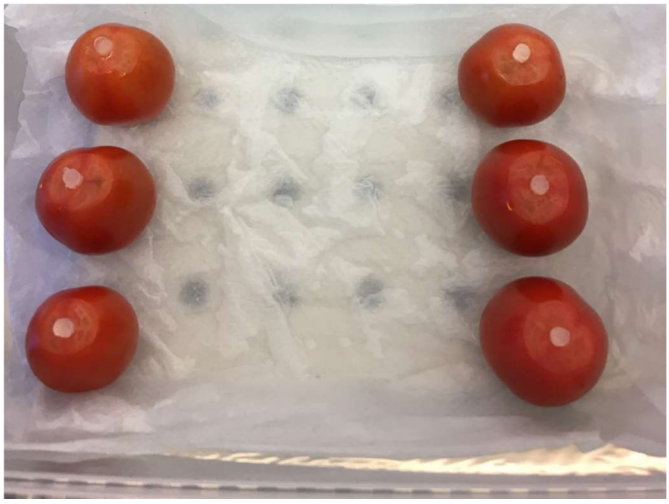

(a)

(b)

Figure 5. *(a) Agar plugs of Fusarium oxysporum placed on the cut-off surface of cherry tomatoes before starting the incubation period; (b) Progression of F. oxysporum growth after 48h of incubation. The three tomatoes on the left were immersed in blank solution while the three tomatoes on the right were immersed in A. vulgaris aquatic extract. Treatment with the plant extract resulted in a visual decrease of the fungal growth.*

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