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Arthropods as vectors of esca-related pathogens: Transmission efficiency of ants and earwigs and the potential of earwig feces as inoculum source in vineyards

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Summary

The spread of Grapevine trunk diseases (GTDs) such as esca concerns wine growers worldwide. Besides rain-splash and air currents, arthropods may play an additional role in the dissemination of esca-related pathogens such as *Phaeomoniella chlamydospora* (*Pch*) and *Phaeoacremonium minimum* (*Pmm*). The present study confirms that black garden ants (*Lasius niger* L., Formicidae: Formicinae) and European earwigs (*Forficula auricularia* L., Dermaptera: Forficulidae) can, under artificial conditions, efficiently transmit spores of *Pch* and *Pmm* to healthy grapevine cuttings, causing new infections.

The potential of earwig feces as inoculum source in vineyards is additionally discussed. Spores of *Pch* and *Pmm* retained germination ability after earwig gut passage, and infectious feces successfully infected wounded grapevine cuttings under artificial conditions. However, molecular detection frequencies of esca-related pathogens in earwig feces collected from the field were very low. With this, the risk of earwig feces as inoculum source for esca-related pathogens is probably only marginal.

However, arthropods carrying esca-related spores on their exoskeletons, such as ants and earwigs, might contribute to the overall spread of esca in vineyards.

The invasion of GTDs during the phase of pruning wound susceptibility, either by arthropod vectors or by airborne spores, can efficiently be prevented by adequate pruning wound protection.

K e y w o r d s : grapevine trunk diseases; *Phaeomoniella chlamydospora*; *Phaeoacremonium minimum*; dispersal; arthropods; earwig feces; transmission efficiency.

Introduction

Arthropods play a role in the dissemination process of many plant diseases. They can transport fungal spores externally on their exoskeleton, or internally after acquirement of spores into the digestive tract (Leach 1940). There are several fungal pathogens known whose spores are externally transported to the pathogen's infection site by arthropods (Leach 1940, Brasier 1991, El-Hamalawi and Menge 1996, Kluth *et al*. 2002). The risk of arthropod feces as inoculum source has also been studied (Evans 1973, Hasan *et al*. 1982, Chen *et al*. 2014), and germination ability of pathogenic spores can even be enhanced after arthropod gut passage (Chen *et al*. 2014).

Studies point out that arthropods are involved in the dispersal of the esca complex, one of the most prominent grapevine trunk diseases (GTDs) that affect grapevine health worldwide (Moyo *et al*. 2014, Kalvelage *et al*. 2021). Causal agents of esca are fungi that colonize the vascular system of grapevines, finally decreasing production and longevity of vineyards (Vasquez *et al*. 2007, Hofstetter *et al*. 2012, Lecomte *et al*. 2012). Typical external symptoms are "tiger-stripe" patterns on leaves, called "Grapevine Leaf Stripe Disease" (GLSD), which is the most prevalent GTD (Mugnai *et al*. 1999, Surico 2009, Mondello *et al*. 2018). Other external symptoms are the wilting of parts or the whole vine called "vine apoplexy" or "acute esca" (Mugnai *et al*. 1999). Internal symptoms include discolorations of the vine's vascular system, known as "brown wood streaking" or Petri disease (Mugnai *et al*. 1999, Mondello *et al*. 2018). In case of white rot present inside the trunk, traced back to the basidiomycetous pathogen *Fomitiporia mediterranea* (Fischer 2002), "esca proper" is denoted. It frequently co-occurs with GLSD in older vineyards (Surico 2009). Initially colonizing pathogens are the ascomycetous fungi *Phaeomoniella chlamydospora* (*Pch*) (Crous and Gams 2000) and *Phaeoacremonium* spp. (*Pm* spp.) (Mostert *et al*. 2006). Esca-related pathogens enter vines mainly through winter pruning wounds (Larignon and Dubos 1997, Mugnai *et al*. 1999, Larignon and Dubos 2000, van Niekerk *et al*. 2011) which are susceptible for several weeks under field conditions (Eskalen *et al*. 2007, Elena and Luque 2016). Additional entry points in the field are sucker wounds resulting from vineyard management (Makatini 2014). Inoculum sources of *Pch* and *Pm. minimum* (*Pmm*), the most prevalent *Pm* spp. in Germany (Fischer and Kassemeyer 2003, Fischer 2019), are found in cracks of the vine trunk (Edwards *et al*. 2001, Rooney-Latham *et al*. 2005b, Baloyi *et al*. 2013). Spores are usually considered as being dispersed by wind or rain splash (Larignon and Dubos 2000, Eskalen and Gubler 2001).

Although Moyo *et al*. (2014) and Kalvelage *et al*. (2021) frequently detected esca-related pathogens on arthropod exoskeletons, the mere presence of potentially infectious spores on arthropod exoskeletons and the activity radius of arthropods does not verify an actual transmission risk.

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However, the attraction of the most prevalent arthropods in South African vineyards to wound sap of freshly pruned vines (Moyo *et al*. 2014) and transmission experiments conducted by Moyo (2013) under experimental conditions demonstrated the potential of ants and millipedes to inoculate healthy grapevine cuttings after exposure to sporulating cultures of esca-related pathogens.

In Germany, European earwigs are, besides spiders, the dominant arthropods on grapevines (Kalvelage *et al.* 2021). Molecular analysis revealed the presence of *Pch* on 27 %, the presence of *Pm* spp. on 17 % and their combination on 9 % of examined earwigs (Kalvelage *et al*. 2021). However, the temporal occurrence of earwigs from the end of May onwards, well outside the immediate phase of pruning wound susceptibility, indicates a more likely transmission risk of earwigs for lately pruned vines only and especially for sucker wounds caused by vineyard management during the vegetation period (Makatini 2014).

Ants, testing positive for *Pch* in 37 %, for *Pm* spp. in 11 % and for their combination in 7 % of the cases, were already present in April, within the presumed susceptibility phase of pruned vines, and were also observed visiting those (Kalvelage *et al.* 2021). Nonetheless, arthropods may rather be considered as incidental vectors dispersing pathogen spores between vines as they migrate during foraging.

With this background, the present study aims to elucidate in more detail the actual risk of arthropod-mediated transmission of esca-related pathogens with following approaches: i) assessment of the *in vitro* transmission efficiency of *Pch* and *Pmm* vectored by ants and earwigs to healthy grapevine cuttings; ii) evaluation of the germination ability of esca-related spores after earwig gut passage; iii) *in vitro* infection of wounded grapevine cuttings with infectious earwig feces; iv) *in vivo* detection of esca-related pathogens in earwig feces collected in vineyards using a nested multiplex PCR approach. Obtained results will help to better define the roles of arthropods in the dispersal process of esca-related pathogens and help to encourage the protection of pruning wounds against pathogen invasion.

Material and Methods

Ant- and earwig-mediated spore transmission of *Pch* and *Pmm* to healthy grapevine cuttings

Arthropod sampling sites: For the transmission experiment, black garden ants (*Lasius niger* L., Formicidae: Formicinae) were collected from different ant trails in the vicinity of the Julius Kühn-Institute (JKI; Siebeldingen, Germany), and European earwigs (*Forficula auricularia* L., Dermaptera: Forficulidae) were collected using cardboard traps (Moyo *et al*. 2014, Kalvelage *et al*. 2021) from an experimental JKI vineyard (49°13'00.2'N 8°02'53.1"E) planted in 1996 with the fungus-resistant (PIWI-) cultivar *Vitis vinifera* 'Phoenix'. Integrated plant protection measures were applied throughout the growing season, and were directed against downy and powdery mildew. Earwigs, with feces to be analyzed by molecular means, were collected in the 'Phoenix'- and a second vineyard (49°13'08.8'N; 8°02'39.6'E) planted in 2002, with the PIWI-cultivar 'Calandro'. Management was organic in the latter. A high occurrence of GLSD-symptoms and apoplexy was documented in the 'Phoenix'-vineyard in the years 2012-2015 (Molnar *et al*. 2020), whereas symptoms were very rare in the 'Calandro'-vineyard.

Cultivation of fungal pathogens: A selected isolate of *Pch* was multiplied on potato dextrose agar (PDA; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) plates for three weeks at room temperature (RT) under daylight conditions. A spore suspension of 15,000 spores·mL^{-1} was prepared using a haemacytometer (Neubauer improved). 100 µL of the suspension were spread each on 18 PDA plates and cultivated for two weeks in an incubator (Cooled Incubator MIR-554-PE, Panasonic, Healthcare Co., Ltd., Gunma, Japan) under continuous white light and 21 °C.

Two compatible isolates were determined for *Pmm* following the procedure described by Mostert *et al*. (2003). After pairing on pine needles for four weeks in an incubator with continuous white light and 21 °C, formation of perithecia representing the teleomorphic state of *Pmm* was observed (Fig. 1A). Both isolates were multiplied on PDA plates and a spore suspension (1 x 10^6 spores·mL^{-1}) was prepared as described above. Six autoclaved pine needles were placed each onto 18 plates containing 2 % H_2O_{dd} agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Spore suspensions of both mating type isolates were pooled and 600 µL equally distributed on the pine needles. Plates were incubated under continuous white light and 21 °C. After approx. eight weeks, spore droplets at the tips of perithecia necks were observed and mature asci with ascospores were found inside (Fig. 1B-D). The cultivation of the two fungal pathogens was repeated four times.

Settings of the transmission experiment: 176 cuttings of *V. vinifera* 'Müller-Thurgau' were hot-water treated for 30 min at 50 °C and then stored at 10 °C for ten days. Three-bud canes were prepared and planted into plastic boxes containing sterile soil. After 5 months of

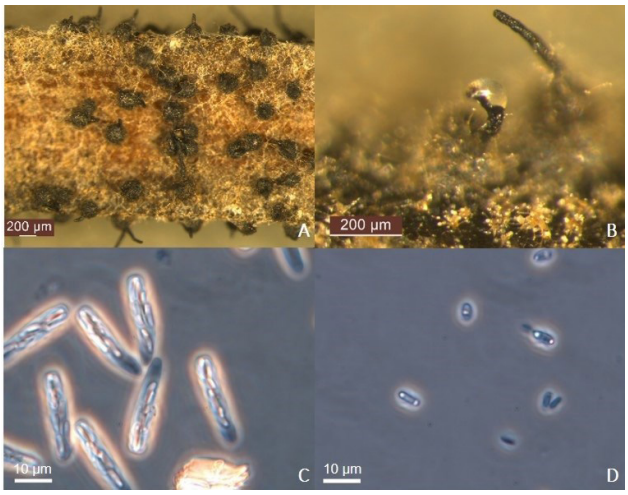

Fig. 1: Perithecia of *Pm. minimum*. Formation of perithecia on pine needles as a result of pairing compatible mating types (**A**); perithecia necks with oozing spore droplet (**B**); asci, each containing eight ascospores (**C**); released ascospores of *Pm. minimum* (**D**).

incubation in the greenhouse, cuttings were placed into pots of 12 cm diameter and cultivated in the greenhouse with 16 °C night- (16 h) and 24 °C daytime (8 h) temperature; relative humidity was 30 %.

Cylinder-shaped plastic cages were constructed from 1 mm thick plastic foil (25 x 30 cm; Moyo 2013; Fig. 2A+B). The pots' surface was covered with foamed plastic placed around the grapevine cutting. Holes of 5 cm diameter at the side of the plastic cages served as portals and were closed with a piece of foamed plastic.

Transmission experiment: The transmission experiment was carried out in August and September 2020. Plates containing either *Pch* or *Pmm*-fruiting structures were placed into plastic trays (6 x 13 x 17 cm). Eight trays were prepared per fungal and arthropod species. Ants were anesthetized for 10 sec with carbon dioxide (CO_2) and aseptically placed in groups of ten into the culture-containing trays (Fig. 2C+D). Due to their small size, ants were used unwashed, whereas earwigs were vortexed for 10 sec in sterile H_2O_{dd} containing 0.01 % Tween®80 (Carl Roth GmbH + Co. KG). After drying on filter paper, earwigs were transferred in groups of ten into the trays containing the particular fungal culture (Fig. 2E). All trays were sealed with punctured lids and stored in a climate chamber (Fitotron® Modular Plant Growth Chambers, Type HGC; WEISS Gallenkamp LTD., Leicestershire, UK) for 24 h at 21 °C with an 8-h-day- and 16-h nighttime rhythm.

Fig. 2: Settings of the transmission experiment: Plastic cage around a grapevine cutting (**A**); earwigs sheltering behind vine leaves (**B**); ants exposed to perithecia of *Pm. minimum* on pine needles (C+D); plastic trays containing arthropods and sporulating fungal cultures on PDA plates (**E**); 3 cm-notch in a grapevine cutting ('Müller-Thurgau') (**F**); cultivation of infected grapevine cuttings in the greenhouse (**G**).

The following day, a 3 cm-notch was aseptically cut into the grapevine stems below the first bud (Fig. 2F). The plastic cages were fixed around the pots and were closed with gauze. Five ants and earwigs per fungal species were aseptically picked from the trays and individually placed into 1.5 mL reaction tubes. They were then freeze-killed for 1 h at -20 °C, before 1 mL of sterile H_2O_{dd} amended with Tween®80 was added. Tubes were vortexed for 60 sec to loosen spores from the exoskeletons. The number of fungal spores (spore load) on the arthropod exoskeleton was then determined using a haemacytometer.

Remaining grouped arthropods were anesthetized with CO_2, aseptically placed inside the cages, and kept inside for 48 h before being aseptically removed.

In each case, a Raspberry Pi 3 Model B V1.2 camera (Raspberry Pi Foundation, Cambridge, UK) was installed for 24 h on one cage containing a wounded grapevine cutting and either ants or earwigs. Images were automatically taken every five sec.

As a positive control, spore suspensions (50,000 spores·mL^{-1}) were generated from *Pch* and *Pmm*-fruiting structures, and 20 µL each placed onto the notch of four grapevine cuttings. As a negative control, 20 µL of sterile water was used.

Two days after arthropod removal, wounds were sealed with laboratory film to avoid evaporation and cross infection. Transmission experiments were repeated four times in four consecutive weeks. All grapevine cuttings were stored in the greenhouse for 12 months with 24 °C day- (8 h) and 16 °C nighttime (16 h) temperature; relative humidity was 30 % (Fig. 2G).

To compare spore loads of arthropods acquired after exposure to sporulating fungal cultures, a generalized linear mixed effect model with poisson distribution and a logarithmic link function was applied with arthropod species, fungal species and their interaction term set as fixed effects and experimental repetition set as random effect. Estimated marginal means (EMMs) with Tukey adjustment were used for the pairwise comparison of groups ($p \leq 0.05$).

Transmission efficiency of arthropods by isolation of target pathogens: After incubation for 12 months, the transmission efficiency of arthropods was determined by isolation of target pathogens from the inoculated grapevines, determining re-isolation rates. The stem was cut 0.5 cm above and below the infected notch. The wooden piece was then dipped into 3 % sodium hypochlorite (NaOCl), followed by 70 % ethanol and sterile H_2O_{dd}, each for 30 sec. After drying on filter paper, samples were flamed for a few seconds.

A longitudinal cut was then aseptically performed through the notch. The bark of the resulting pieces was flamed again, small pieces (approx. 0.5 x 0.5 cm) were cut from the necrotic tissue, and ten pieces per sample placed on two PDA plates supplemented with chloramphenicol (5 mg·L^{-1}).

Plates were monitored for up to four weeks. Fungal colonies with the appearance of *Pch* and *Pmm* were subcultured and identified using morphological characteristics (Crous *et al.* 1996, Crous and Gams 2000). Transmission of *Pch* and *Pmm* to wounded grapevine cuttings by ants or earwigs was classified as successful when the respective fungus was isolated.

To compare the transmission efficiency, a generalized linear model assuming quasibinomial distribution was applied with arthropod species, fungal species, experimental repetition and their interaction terms set as fixed effects. EMMs with Tukey adjustment were used for the pairwise comparison of groups ($p \leq 0.05$). In order to compare re-iso-

lation rates of spore inoculated control plants, a two-sample t-test was applied ($p \leq 0.05$).

Germination ability of fungal pathogens after earwig gut passage

Feeding of earwigs on grapevine pathogens: *Pch* and *Pmm* were grown on three PDA plates each and cultivated for two weeks in a climate chamber at 21 °C and 8-h day- and 16-h nighttime rhythm.

Earwigs, collected from the 'Phoenix'-vineyard, were placed in groups of five into 50 mL tubes, and then starved for 24 h before being surface sterilized by vortexing for 10 sec in 10 mL of NaOCl supplemented with 0.01 % Tween®80. Earwigs were aseptically picked, briefly dried on filter paper, and transferred into new 50 mL tubes. After vortexing for 10 sec in 10 mL of H_2O_{dd} supplemented with 0.01 % Tween®80 to remove residual NaOCl, they were again dried on filter paper.

Plates containing fungal cultures were positioned without lid in a plastic tray (6 x 13 x 17 cm) and five surface sterilized earwigs were added (Fig. 3A). Three replicates were prepared for each fungal pathogen. Three boxes, each with five earwigs feeding on empty PDA plates, were used as negative control. All boxes were closed, covered with black foil to comfort the nocturnal earwigs, and kept for 18 h in a climate chamber at 21 °C with an 8-h day- and 16-h nighttime rhythm. Images of feeding traces on fungal cultures were taken after the feeding experiment.

Surface sterilization and collection of fecal pellets: After 18 h of feeding (Fig. 3B), earwigs were surface sterilized with NaOCl and sterile H_2O_{dd} supplemented with 0.01 % Tween®80. Earwigs from one box were aseptically picked, placed into a sterile 50 mL tube and vortexed in 10 mL NaOCl and 10 mL of sterile H_2O_{dd}, each for 10 s. Between vortexing steps, earwigs were briefly dried on filter paper. Washing steps were repeated, and earwigs were placed into sterile 50 mL tubes to recover. After 1 h, earwigs from one tube were aseptically picked, placed into a new 50 mL tube containing 10 mL of sterile H_2O_{dd}, and vortexed for 30 sec. After drying on filter paper, earwigs were placed in groups of five for 24 h into clean plastic trays. The last washing suspension was centrifuged for 10 min at 10,000 rpm, the supernatant was discarded and approx. 2 mL kept of which 100 µL each were spread on three PDA plates supplemented with chloramphenicol (5 mg·L^{-1}). Growth of fungal cultures was monitored for two weeks at RT, and the number of colony forming units (CFUs) was determined. This procedure served as control of the sterilization efficiency.

After 24 h fecal pellets of earwigs from the same tray were pooled in 2 mL reaction tubes. Two steel beads and 500 µL of sterile H_2O_{dd} were added, and a suspension was generated by placing the samples for 30 sec in a tissue lyser (Tissue Lyser 2, Qiagen, Hilden, Germany) at 30 Hz. Fecal suspensions were diluted with sterile H_2O_{dd} in 10-fold steps from 10^{-1} to 10^{-5}. In order to identify single target colonies, 100 µL of each dilution were individually spread on a PDA plate supplemented with chloramphenicol (5 mg·L^{-1}) and the number of CFUs of the target species was determined after two weeks at RT. The whole feeding experiment was repeated four times.

Germination ability after earwig gut passage was compared between the pathogens using a two-sample t-test ($p \leq 0.05$).

In vitro infection of grapevine wounds with infectious earwig feces

Preparation of infectious earwig feces: Cultures of *Pch* and *Pmm* were prepared and earwigs collected from the field as described above. Ten earwigs each were placed into a plastic tray containing a particular fungal culture. Earwigs were allowed to feed for 24 h. For each fungus, ten replicate trays were prepared. Trays containing ten earwigs and empty PDA plates served as negative control. After feeding, earwigs were surface sterilized as described above and placed into clean plastic trays. After 18 h, fecal pellets from the same fungus were pooled in 2 mL reaction tubes. A fecal suspension was created with approx. 2.2 mg of feces and 250 µL of sterile H_2O_{dd}. Two steal beads were added and the suspension was homogenized for 30 sec in a tissue lyser at 30 Hz.

Infection of grapevine cuttings with fecal suspensions: Cuttings of *V. vinifera* 'Müller-Thurgau' were prepared as described above and 20 µL of the fecal suspensions were placed onto the 3-cm notch. Eight replicates were prepared for each fecal-fungus combination. Four grapevine cuttings inoculated with 20 µL of either a *Pch* or a *Pmm* spore suspension (5,000 spores·mL^{-1}) served as positive, and four grapevine cuttings inoculated with 20 µL of sterile H_2O_{dd} as negative control. Inoculated wounds were sealed with laboratory film after 2 d in the greenhouse. Plants were incubated in the greenhouse

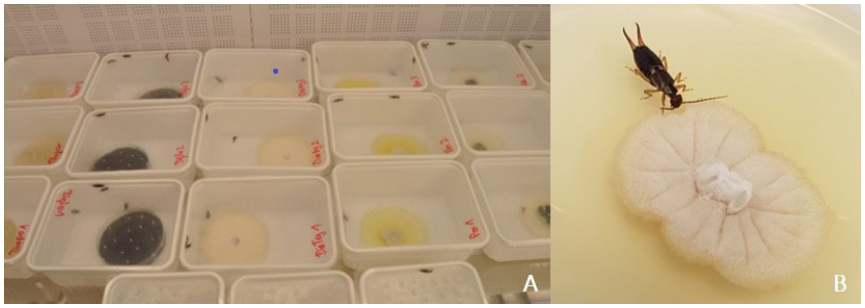

Fig. 3: Plastic trays containing five earwigs feeding on esca-related pathogens (**A**); earwig feeding on a culture of *Pm. minimum* (**B**).

for 7 months with 16 °C night- (16 h) and 24 °C daytime (8 h) temperature; relative humidity was 30 %. The number of viable spores in the fecal suspensions was determined by spreading 100 µL from a dilution series (10^{-1} to 10^{-4}) onto PDA plates supplemented with chloramphenicol (5 mg·L^{-1}). Number of CFUs of the target species was determined after two weeks at RT.

Examination of the infection potential: The infection potential of earwig feces was evaluated after seven months by isolation of target pathogens from the inoculated grapevine cuttings as described above. The infection was considered successful when the particular fungus was isolated from the grapevine cutting.

Molecular detection of esca-related pathogens in earwig feces collected in vineyards

Collection of earwig feces: Earwigs were collected from June to August 2021 in the 'Phoenix'- and 'Calandro'-vineyards. Nine cardboard traps per vineyard were wrapped around different vine trunks (Moyo *et al*. 2014, Kalvelage *et al*. 2021). Every other week, ten randomly chosen earwigs from each trap were aseptically picked and pooled in 50 mL tubes. Earwigs from the same tube were surface sterilized by vortexing in 15 mL NaOCl and sterile H_2O_{dd} amended with 0.01 % Tween®80, each for 10 sec. After the first washing step, earwigs were dried on filter paper and placed into new 50 mL tubes. After the second washing step, earwigs were dried on filter paper and placed into clean plastic trays (6 x 13 x 17 cm). Earwigs were released from the boxes after 24 h and fecal pellets of three consecutive trays were aseptically pooled into 2 mL reaction tubes.

Molecular detection of esca-related pathogens in earwig feces: DNA was isolated from earwig feces using the DNeasy® UltraClean® Microbial Kit (Quiagen) as advised by the manufacturer. DNA was then subjected to the nested multiplex PCR approach as described in Kalvelage *et al*. (2021) to detect esca-related pathogens. PCR products were analyzed by loading onto a 2 % agarose gel and running at 6 V/5 cm for 1.5 h. A QUANTUM ST5 gel documentation system (Vilber Lourmat, Eberhardzell, Germany) visualized the PCR products under ultraviolet (UV) light.

Statistical analyses: All statistical analyses were conducted using the R software v 3.6.1 (R Core Team 2021) and the R packages *ggplot2* v 3.3.3 (Wickham 2016), *dplyr* v 1.0.2 (Wickham *et al.* 2020), *lme4* v 1.1-21 (Bates 2015), *emmeans* v 1.5.4 (Lenth 2021) and *multcomp* (Hothorn 2008).

Results

Transmission efficiency of ants and earwigs vectoring *Pch* and *Pmm* to healthy grapevine cuttings

Ants and earwigs on artificial wounds: Camera recordings capturing ants and earwigs for 24 h inside the transmission cages revealed their activity both at day- and nighttime (Fig. 4). Ants were mostly active on the foamed plastic covering the soil, but also visited the wound at day- (Fig. 4A) and nighttime (Fig. 4B+C). The nocturnal earwigs sought shelter behind vine leaves during daytime (Fig. 4D), but also visited the wound (Fig. 4E), preferably during nighttime (Fig. 4F). Both arthropods were observed drinking from the plant sap accumulating on the wounds.

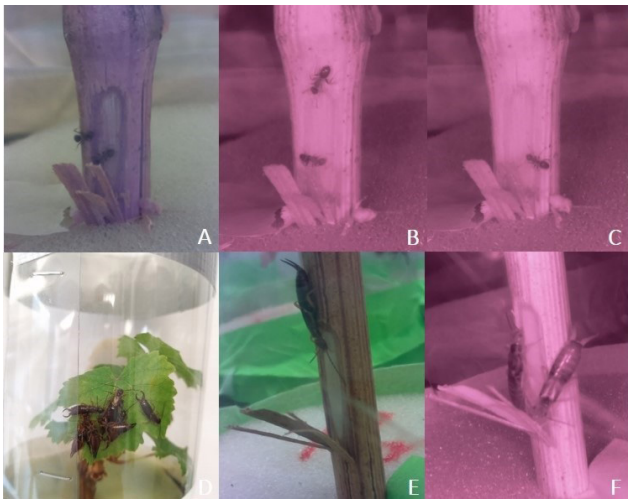

Fig. 4: Activity of arthropods on grapevine cuttings during the transmission experiment: Ants visiting the fresh wound and drinking on wound sap during day- (**A**) and nighttime (**B**+**C**); earwigs seeking shelter behind leaves during daytime (**D**); earwigs visiting the fresh wound at day- (**E**) and nighttime, drinking on wound sap (**F**).

Spore loads of ants and earwigs after exposure to sporulating fungal cultures: The amount of *Pch* and *Pmm* spores on exoskeletons of five randomly chosen ants and earwigs after exposure to the respective cultures was determined for each of the four repetitions of the transmission experiment. In general, both arthropods acquired significantly more spores of *Pch* than *Pmm* on their exoskeletons (Tab. 1).

The significantly largest number of spores was found on earwigs carrying *Pch*. The sporeloads of earwigs carrying *Pmm* and ants carrying *Pch* did not significantly differ and the significantly lowest number of spores was found on ants carrying *Pmm*.

Transmission efficiency of ants and earwigs: Re-isolation rates from grapevines infected with spore suspensions were 31.25 % ± 6.25 (SE) for *Pch* and 75.00 % ± 10.21 (SE) for *Pmm*. Differences were significant (t-test, $p = 0.011$). No target pathogen was isolated from water-inoculated wounds.

Both ants and earwigs efficiently transmitted esca-related spores to wounded grapevine cuttings, eventually leading to infection. The average re-isolation rate of *Pch* vectored by ants was 21.88 % ± 5.98 (SE) and 12.50 % ± 5.10 (SE) when vectored by earwigs. The average re-isolation rate of *Pmm* vectored by ants was 37.50 % ± 14.43 (SE) and 46.88 % ± 3.13 (SE) for earwigs.

No significant effects of experimental repetition or arthropod vector was found, but the fungal species had a significant impact on the transmission efficiency ($p \leq 0.0055$). The

Table 1

Spore loads of ants (*Lasius niger*) and earwigs (*Forficula auricularia*) after exposure to sporulating cultures of *Pch* and *Pmm* and statistical analysis using a pairwise Tukey-test ($p \leq 0.05$)

Arthropod	Pathogen	Mean number of spores ± SE	95 % Confidence interval	Significance
F. auricularia	*Pch*	2,229,688 ± 878,243	1,275,295 – 3,897,531	a
F. auricularia	*Pmm*	178,750 ± 111,941	70,065 – 214,133	b
L. niger	*Pch*	122,500 ± 13,769	87,907 – 267,151	b
L. niger	*Pmm*	32,500 ± 24,875	15,983 – 48,574	c

Table 2

Re-isolation rates (transmission efficiency) of esca-related pathogens vectored by arthropods to grapevine cuttings and statistical analysis using the pairwise Tukey test ($p \leq 0.05$)

Vectored pathogen	Mean re-isolation rate ± SE [%]	95 % Confidence interval	Significance
Pch	17.19 ± 4.05	8.11 - 29.6	a
Pmm	42.19 ± 7.06	28.74 - 56.0	b

infection potential of *Pch* vectored by arthropods was found to be significantly lower in comparison to *Pmm* (Tab. 2).

Germination ability of esca-related pathogens after earwig gut passage

In vitro feeding-experiment: Earwigs, starved for 24 h, fed both on *Pch* and *Pmm* cultures (Fig. 5). However, not all individuals fed on the cultures.

Viability of esca-related spores after earwig gut passage: After earwig gut passage, conidia of *Pch* and *Pmm* were able to germinate. No target pathogens developed from feces of earwigs that fed on empty PDA plates. Cumulated over the four replications, *Pch* colonies formed on 10 out of 12 plates and *Pmm* on 8 out of 12 plates. The relative germination ability did not significantly differ between examined pathogens (two-sample t-test, $p = 0.2380$; Tab. 3). The number of CFUs was calculated for all plates (spores·mL^{-1}) and revealed a mean

Table 3

Germination ability of esca-related pathogens after earwig gut passage and statistical analysis using a two-sample t-test ($p \leq 0.05$)

Fungus	Mean germination ± SE [%]	95 % Confidence interval	Significance
Pch	91.67 ± 8.33	47.2 - 99.4	a
Pmm	66.67 ± 13.61	33.0 - 89.4	a

spore number of 1.5 x 10^6 ± 9.4 x 10^5 (SE) spores·mL^{-1} for *Pch* and 7.7 x 10^6 ± 4.5 x 10^6 (SE) spores·mL^{-1} for *Pmm*.

Effectiveness of the earwig exoskeleton sterilization process: The removal of fungal spores from earwig exoskeletons was verified. Despite post-feeding sterilization, 4 out of 12 plates showed growth of *Pch* and 6 out of 12 plates showed growth of *Pmm*. The number of CFUs was calculated for all plates and revealed a mean number of 164 ± 139 (SE) spores·mL^{-1} for *Pch* and 363 ± 221 (SE) spores·mL^{-1} for *Pmm*. In comparison to the number of CFUs calculated from feces of earwigs that fed on sporulating cultures, mean number of spores retaining viability after sterilization process was much lower.

Artificial infection of grapevines using infectious earwig feces

The concentration of the fecal suspensions used to inoculate grapevines was approx. 4,500,000 spores·mL^{-1} for *Pch* and approx. 350,000 spores·mL^{-1} for *Pmm.* Both pathogens were isolated from wounds inoculated with in-

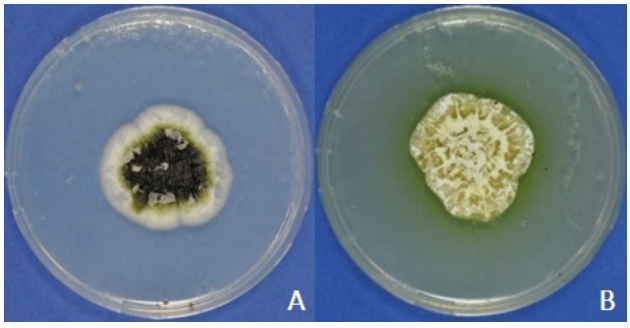

Fig. 5: Feeding traces of earwigs on *Pa. chlamydospora* (**A**) and *Pm. minimum* (**B**).

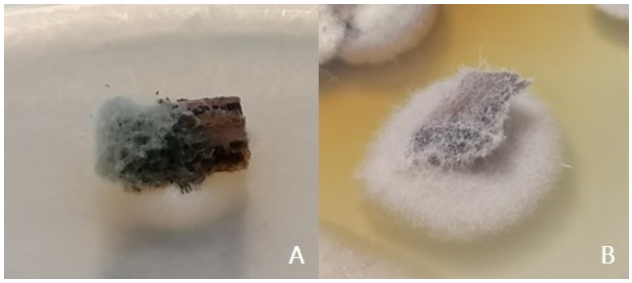

Fig. 6: Outgrowth of *Pa. chlamydospora* (**A**) and *Pm. minimum* (**B**) from grapevine-wound tissue inoculated with infectious earwig feces.

fectious earwig-feces (Fig. 6). Re-isolation rates from the eight vines inoculated with earwig feces were 75 % for *Pch* and 63 % for *Pmm.* From the four positive control plants, inoculated with spore suspensions, *Pch* was recovered in 75 % and *Pmm* in 100 % of the cases. No target pathogens were isolated from water-inoculated wounds.

In vivo investigation of earwig feces for esca-related pathogens using molecular techniques

In order to determine the risk of earwig feces as inoculum source for esca-related pathogens, DNA of earwig feces collected from vineyards, was subjected to the nested multiplex PCR approach (Kalvelage *et al*. 2021). Only in two out of 36 pooled samples *Pch* was detected, minimizing the risk of earwig feces as inoculum source.

Discussion

Esca-related pathogens have been detected on exoskeletons of diverse arthropods found on grapevine trunks indicating involvement in the dispersal of the disease (Moyo *et al*. 2014, Kalvelage *et al*. 2021). However, the mere presence of potentially infectious spores on arthropod exoskeletons and the activity radius of arthropods on grapevines does not result in an actual transmission risk.

Results of the present study confirm transmission of *Pch* and *Pmm* spores vectored by ants and earwigs to healthy grapevine cuttings. Arthropod-mediated transmission was significantly more efficient for *Pmm* (42.19 %) than for *Pch* (17.19 %). This relation is consistent with re-isolation rates obtained from positive control plants, where *Pmm* (75.00 %) was statistically more frequently re-isolated than *Pch* (31.25 %). However, control plants were inoculated with 20 µL suspensions containing approx. 1,000 spores, while arthropods carried greatly varying numbers of spores on their exoskeleton, with 640,860 spores on average. How many of these were actually deposited in the wounds is unclear, however, and germination rate possibly plays another role. The number of spores on arthropod exoskeletons was found to be significantly influenced by the involved arthropod and fungal species. The higher spore numbers on earwig exoskeletons in comparison to ants can easily be explained by the particular arthropod size. Average *Pch* spore loads were significantly higher than *Pmm* spore loads on both arthropods after exposure to sporulating cultures. This might be explained by a reduced number of spores produced by the teleomorphic fruiting structures restricted to pine needles in comparison with *Pch* cultures used in the present experiment that covered the entire PDA plate. However, perithecia of *Pmm* seemed morphologically very suitable for arthropod-mediated spore dispersal (Mostert *et al*. 2003, Rooney-Latham *et al*. 2005a, Mostert *et al*. 2006), wherefore experiments were conducted with these fruiting structures.

Transmission efficiency of *Pch* vectored by ants (21.88 %) was in accordance with Moyo (2013) who isolated *Pch* from 8 out of 30 plants (27 %) inoculated by *Pch*-carrying ants. In contrast to ants, the present study is the first to show that earwigs can also efficiently transmit esca-related spores to grapevine wounds, causing new infections.

Both fungal species grow very slowly on artificial media and overgrowth by co-isolated fungi generally impairs target pathogen identification. Our *Pch* isolate was found to grow even slower than *Pmm* (data not shown), which might explain the low isolation rate obtained from positive control plants. Consequently, actual re-isolation rates of arthropod-inoculated plants might have been somewhat higher.

Both arthropods acquired large spore numbers on their exoskeletons during the transmission experiment but these artificial conditions might be different from those in the vineyard. There, arthropods can acquire inoculum from the pathogens sporulating in crevices of the trunk wood (Edwards *et al*. 2001, Rooney-Latham *et al*., 2005b, Baloyi *et al.* 2013). In order to compare *in vitro* and *in vivo* conditions, assessment of the actual spore numbers on arthropods derived from the field is crucial. Only this will finally allow to more accurately estimate transmission efficacy in vineyards. In addition, attention should be paid to abiotic conditions, such as temperature, moisture, humidity or light, influencing germination ability of fungal spores (Hong *et al*. 1997), and, thus, transmission efficiency.

Black garden ants (*L. niger*), captured from ant trails to be examined in the transmission experiment, were also frequently found on vine trunks in Germany (Kalvelage *et al*. 2021). Involvement of ants in the dissemination of esca has been previously described (Moyo *et al*. 2014). In German vineyards, ants frequently tested positive for esca-related pathogens and visited wounds as early as April when late-pruned vines are still susceptible to some degree (Kalvelage *et al*. 2021). Though an attraction of ants to wound sap of vines has, in contrast to South African observations, not been noticed in German vineyards (Moyo *et al*. 2014), ants during the transmission experiment drank from the wound sap that accumulated on the fresh wounds.

European earwigs are the predominant arthropod species in German vineyards and esca-related pathogens were frequently detected on their exoskeletons (Kalvelage *et al*. 2021). In addition to their potential as external vector, the risk of earwig feces as inoculum source in vineyards is discussed in the present study. Moyo *et al*. (2014) already revealed the ability of *Pch* spores to regrow from millipede feces. Our results show that the omnivorous earwigs fed on the sporulating cultures they were exposed to, and spores of *Pch* and *Pmm* showed a germination rate of 91.97 % and 66.67 %, respectively, after gut passage. Although the sterilization of earwig exoskeletons after feeding did not eliminate all spores, the numbers of CFUs calculated after plating washings from sterilized earwigs were much lower for *Pch* and *Pmm* in comparison to CFU numbers calculated after cultivation of infectious earwig feces. Consequently, spores of both pathogens retained germination ability and were able to regrow from earwig feces. The high variation regarding the number of CFUs of spores germinating from feces can be explained by the irregular feeding of earwigs on the fungal cultures.

Our results show successful inoculations of healthy grapevine cuttings with earwig feces containing spores of *Pch* or *Pmm*. However, even though earwigs fed on the

particular fungal cultures under artificial conditions with no other food source provided, in only two out of 36 fecal samples, originating from earwigs collected in experimental vineyards, *Pch* was detected using molecular techniques. These results may indicate a low risk of earwig feces as inoculum source for esca-related pathogens. Omnivorous earwigs can find various food sources in the field and dissection of earwigs from various sites revealed moss, grass, lichen and aphids as common foods (Crumb *et al*. 1941). Mycelium or spores of *Pch* and *Pmm* do not seem to be preferred as food source under natural conditions.

Results of the present study highlight the potential risk of arthropods as external vectors for the esca disease. Nonetheless, as no particular attraction of arthropods towards pruning wounds was noted in German vineyards and most pruning wounds are no longer susceptible when arthropods become active after winter time, they rather may be considered as incidental vectors, visiting pruning or sucker wounds during foraging. With this, however, they contribute to the spatial spread of inoculum in the field. In contrast to winter pruning wounds, summer wounds, *i.e.*, sucker wounds, may play an important role as pathogen portals (Makatini 2014) and should be further examined. Especially earwigs, active in German vineyards from the end of May onwards (Huth 2011, Kalvelage *et al*. 2021), might represent a threat to sucker wounds.

Overall, the phase of pruning wound susceptibility needs to be closely considered when wound protection against arthropods is planned. The phase of susceptibility under different conditions such as climate, cultivar, or pruning measures has not yet been carefully evaluated (Eskalen *et al*. 2007, Elena and Luque 2016) but data would be crucial to give detailed advice for pruning time and approach, considering both air- and arthropod-borne pathogens.

The invasion of grapevine trunk diseases, either by arthropod vectors or by airborne spores, can most likely be prevented by adequate pruning wound protection.

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Conflict of interest

The authors declare no conflict of interest.

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