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Originalarbeit

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Spatial patterns of microsclerotia from *Verticillium dahliae* Kleb. in soils of Bavarian maple stands

Die Verbreitung von Mikrosklerotien des Erregers *Verticillium dahliae* Kleb. in den Böden bayerischer Ahornbestände

Abstract

During the last years, the Bavarian State Institute of Forestry received increasing numbers of announcements that dealt with necrosis on stems of maple trees caused by the soilborne fungus *Verticillium dahliae* Kleb. In this study, five forest areas were investigated along transects up to 100 m by soil sampling and mapping of diseased trees to find out if the pathogen had been introduced by infested maple seedlings. Our results yielded no evidence for this assumption. The spatial distribution of microsclerotia concentrations in the soil did not correlate with spatial patterns of diseased trees. This indicates that *Verticillium* was established on the sample plots independently from healthy or infested maple trees. Counting of year rings of infested maple trees revealed that the trees had developed their necrosis immediately after the drought in the year 2003. Hence, a connection between these extreme climate conditions and disease incidence cannot be excluded.

Key words: Acer, Verticillium, stem canker, microsclerotia, drought, nekrosis, soilborne pathogen

Zusammenfassung

In den vergangenen Jahren wurden durch die Bayerische Landesanstalt für Wald und Forstwirtschaft vermehrt Stammnekrosen an Ahornbäumen, die durch den bodenbürtigen Erreger *Verticillium dahliae* Kleb. verursacht werden, diagnostiziert. In dem hier vorgestellten Projekt wurden in fünf Ahornbeständen entlang eines jeweils 100 m langen Transektes Bodenproben auf Mikrosklerotien hin und Stammschäden an dem Bestand untersucht. Ziel war es zu prüfen, ob der Erreger durch infiziertes Pflanzgut in die Bestände gebracht worden ist. Die Ergebnisse konnten diesen Zusammenhang nicht bestätigen. Die Verteilung der Mikrosklerotien im Boden wies keinen erkennbaren Zusammenhang mit den geschädigten Ahornbäumen auf. Die Ergebnisse deuten viel mehr darauf hin, dass sich der Erreger unabhängig von gesunden und kranken Ahornen in den Beständen etabliert hat. Jahrringzählungen anhand von geschädigten Bäumen deuten darauf hin, dass der Schaden unmittelbar nach der extremen Trockenheit im Jahr 2003 eingetreten ist. Somit wird ein Zusammenhang zwischen extremen Witterungsereignissen und der Erkrankung vermutet.

Stichwörter: Acer, Verticillium, Stammnekrosen, Mikrosklerotien, Trockenheit, bodenbürtige Pathogene

Introduction

The soilborne fungus *Verticillium dahliae* Kleb. is a polyphageous plant pathogen responsible for wilt diseases of many economically important plants mainly in the temperate and subtropical climates (Pegg and Brady, 2002). Its host range includes herbaceous plants and crops as well as shrubs and trees. Typical symptoms on maple trees are wilting followed by dieback of single branches or parts of the crown. Some infected trees exhibit stem necrosis with

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a typical greenish colour and lengths ranging from few centimetres to three or four metres (Schneidewind, 2005, Fig. 1). In some cases, secondary damages by wood decomposing fungi or wood breeding beetles can lead to stem breaking.

When the colonized tissues die, *Verticillium dahliae* produces microsclerotia to survive outside a host (Neubauer et al., 2009). After the decomposition of dead plant tissues, these microsclerotia are released into the soil, where they can persist up to 14 years (Wilhelm, 1955). Increasing amounts of dead plant material remaining on an area, as for example crop sequences with potato cultivation, result in a massive accumulation of microsclerotia in the soil (Neubauer and Heitmann, 2011).

Germination of microsclerotia is triggered by root exudates from potential hosts (Schreiber and Green, 1963; Olsson and Nordbring-Hertz, 1985). After infection via the elongation zone of young roots or wounds, *Verticillium* enters the xylem vessels (Butin, 1996; Schneidewind, 2005). By hyphal growth and the release of conidia, the pathogen spreads inside the plant (Sinclair et al., 1989). The wilting symptoms are caused by a tracheomycosis. The disease can spread by transportation of microsclerotia in fallen leaves, seeds of host plants or any other infected host tissue. Infected planting material and contaminated soil transported with nursery plants may also contribute to the dissemination of the pathogen in forest stands (Rijkers et al., 1992).

Fig. 1. Stem necrosis on a Norway maple caused by *Verticillium dahliae* (Photo: A. Nannig).
Stammnekrosen an Bergahorn verursacht durch Verticillium dahlia (Foto: A. Nannig).

Highly susceptible forest tree species native to Germany are Sycamore (*Acer pseudoplatanus* L.), Norway maple (*A. platanoides* L.) and Field maple (*A. campestre* L.). During the last years, the number of announcements of maples showing stem necrosis that reached the Bavarian State Institute of Forestry increased noticeably (Siemonsmeier et al., 2010). Our laboratory cultures proved the presence of *Verticillium dahliae* in the necrotic tissue. In Austria, similar tendencies were observed since 1995 (Demel, 2002). First investigations in the reported areas revealed that the disease is more common in Bavarian forests than formerly known. The age of affected maple trees ranged from 7 to 65 years-old with a mean of 20 years-old. The disease was present on mainly loamy and moderately dry to fresh soils. Examined areas were flat or slightly sloped with different expositions except one stock located on the bottom of a creek valley with steep slopes.

In a further study, we examined five of the announced plots in Bavarian forests for the spatial patterns of *Verticillium* microsclerotia concentration in the soil to find out if the pathogen had been introduced to the plots by planting infected maple seedlings or if it was established all over the area independently from diseased trees. For this reason, a transect was placed in each plot and the quantity of *Verticillium* propagules in soil samples was determined every four metres. Afterwards, the trees up to ten metres distance from the transect were mapped and the occurrence of typical necrosis for each tree noted. In one of the five plots, five trees were felled in order to determine the year of disease incidence by counting of year rings and to confirm the presence of *Verticillium dahliae* by laboratory cultures.

Materials and Methods

Sample areas

All five sample areas were located in Bavaria, Germany (Fig. 2). Plot number 1 was an area in Kranzberg Forest near Freising. Soil was fresh and loamy. The area was flat, being located on the bottom of a small valley. The trees had an age of approximately 15 years-old. Plot number 2 was a flat riparian forest near Ettenbeuren. The maples were approximately 20 to 30 years old. Plot number 3 was a former field slightly tilted to north-western direction near Falkenstein in the Bavarian Forest. The trees had an age of 7 years-old. Soil type was a fresh to slightly semi-humid loam. Plot number 4 was a flat, former field near Meitingen with moderately fresh to moderately dry fine loam. The age of the trees was 16 years. Plot number 5 was located near Geisfeld. The area was sloped in northern direction. Soil was a moderately fresh loam. The southern part of the site was a former pinewood, whereas the northern part was an afforestation of farmland. Planted trees were 25 years old. All investigated maple stockings had been planted, except in plot number 5,

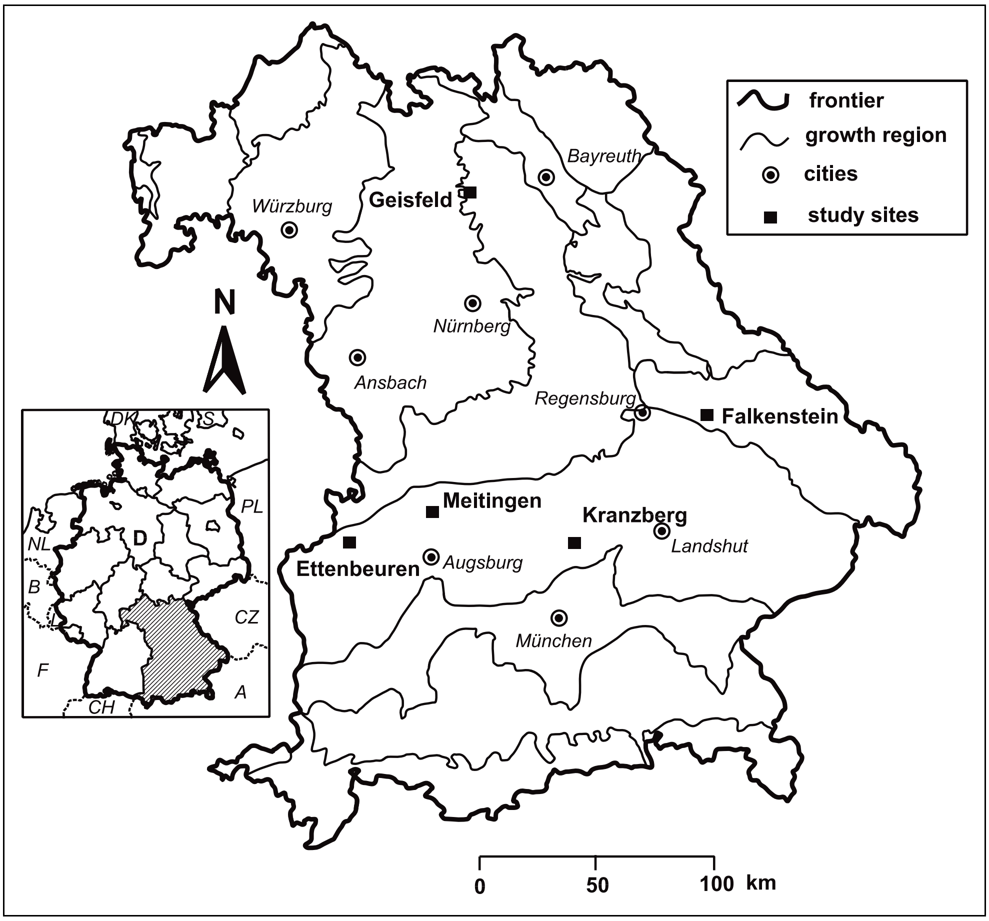

Fig. 2. **Map of Bavaria with the position of the five sampled areas.** *Verteilung der Versuchsflächen in Bayern.*

where wild Field maples grew in a Norway maple planting.

Soil sampling and mapping

For each plot, a transect length of 100 m was intended. Plots number 3 and 5 were too small for a 100 m transect, so the length was reduced to the maximum possible (64 m for plot 3 and 80 m for plot 5). The transects were marked by a rope with a picket at both ends. Soil samples were taken every 4 m, beginning at transect position zero. The samples were obtained with a soil core of 2.5 cm diameter to a depth of 40 to 60 cm and collected separately in polyethylene bags. The soil core was cleaned and disinfected after every sample to avoid contaminations. Mapping of the trees on both sides of the transect line was done up to a distance of 10 m from the transect. For each tree inside this area, the distance to the transect, the corresponding transect position, the tree species and the occurrence of necrosis in case of a maple species were noted.

Isolation of Verticillium dahliae from soil samples

Analysis of soil samples was done according to Harris et al. (1993). In laboratory, the soil samples were taken into open plastic boxes and dried in drying chambers at 23°C for 2 weeks. Samples that could not be homogenized by hand after drying because of high clay contents were pestled until sieving through a 2 mm mesh. Twenty five g of the sieved soil were weighed into an Erlenmeyer flask and made up with 100 ml distilled water. This suspension was placed on a rotary shaker with 300 oscillations per minute to break the aggregates in the soil sample. After 1 h of shaking, the soil suspension was washed first through a 160 µm sieve and afterwards through a 20 µm sieve. The residue on the 20 µm sieve was rinsed with 100 ml distilled water into a glass petri dish. Two ml of this soil solution were then plated on a selective medium according to Heppner (1995). Five replications were done for each soil sample. After 3 weeks of incubation at room temperature in the dark, the soil particles were carefully removed from the agar by rinsing with tap water. Counting of *Verticillium* colonies was done with a binocular loupe. One colony represented at least one germinated microsclerotium from the soil solution (Neubauer et al., 2007). The number of microsclerotia per g dried soil was calculated with the mean value over five replications per sample. Calculation of Lloyd's Index of Patchiness (LIP) (Campbell and Madden, 1990) was done to analyze spatial patterns of microsclerotia in the five sample areas.

Counting of year rings

In plot number 1 (Kranzberg), five randomly chosen trees with stem necrosis were felled to obtain stem discs from necrotic areas. Stem discs were sliced with a band saw. In laboratory, the face of the stem discs was cut with a razor blade to get a plain surface. In order to determine the year of stem colonization, just year rings that had been built up after formation of stem necrosis were counted. For each tree, two stem discs were examined. Counting of year rings was done with a binocular loupe.

Isolation of Verticillium dahliae from necrotic woody tissue
For the isolation of *Verticillium dahliae*, pieces were cut from the greenish necrotic sapwood and surface sterilized with 70% Ethanol. Afterwards, they were washed twice in distilled water und then placed on malt extract agar. Developing colonies were identified by microscopical investigation.

Results

The mapping of trees showed that infected maples were in most cases randomly distributed. In the first plot, in Kranzberg Forest, the number of diseased trees declined from the eastern end of the transect to the western end. The other four plots showed no gradient or clusters in the spatial distribution of diseased maple trees. Among the three maple species that occurred in the five plots, Sycamore was the most common tree as it was in four out of five plots the main tree species. Norway maple was main tree species in one plot and added to Sycamore in three plots. Field maple occurred in low numbers only in the plot with Norway maple as main tree species. The percentage of diseased trees varied at the different plots as well as among the three maple species. Over all plots, the rates of infested trees were 30% for Sycamore, 43% for Norway maple and 39% for Field maple. The percentage of diseased trees on single plots is shown on Tab. 1.

The concentrations of microsclerotia in soil samples along the transects varied at the different plots as well (Fig. 3). Values for individual samples ranged from 0 to 52 microsclerotia in one plot. Mean values are given in Tab. 1. The analysis of microsclerotia concentrations versus the distances from the soil sample positions to the next diseased tree, respectively, yielded no significant correlation (Fig. 4). The direct neighbourhood of an infected tree did not result in higher microsclerotia concentrations in the particular soil sample. Mean values for microsclerotia concentrations in all soil samples of one plot showed high standard deviations, representing the strong variation of propagule densities even over a distance of 4 m (data not shown). Calculation of Lloyd`s Index of Patchiness (LIP) revealed random patterns of propagule densities in plots number 1, 2 and 4 with LIP estimates less than 1, whereas the distribution was aggregated in plots 3 and 5 with LIP values greater than 1 (Tab. 1).

Counting of year rings on stem discs from five diseased trees felled in plot number 1 showed that four out of five trees had developed their necrosis after the growing season in 2003. Just one tree developed his necrosis in 2004. In a few samples, little necrotic spots could be observed in the first healthy year ring after the formation of necrosis, but they were rare and had obviously not spread further. Isolation of the fungus from necrotic tissue was successful.

Tab. 1. Results of soil sampling and mapping
Ergebnisse der Bodenuntersuchungen und Anteil der befallenen Bäume

Sample area	Infested trees [%]	Micro-sclerotia/ g soil	LIP
Kranzberg	21,5	11,2	0,98
Ettenbeuren	24,3	9,7	0,86
Meitingen	40,9	8,1	0,64
Falkenstein	24,7	7,1	1,69
Geisfeld	55,7	13,7	1,19

Discussion

The objective of this study was to answer the question if the disease of maple trees in the five plots was introduced to the areas by planting infected seedlings or seedlings growing in infested nursery substrate. In this case, high microsclerotia concentrations in soil samples close to infected trees were expected as was shown by Schneidewind (2005). However, the results of both mapping and soil sampling yielded no evidence for the introduction of *Verticillium* through infected plant material or infested nursery substrate. Although the mean microsclerotia concentrations on the plots were high and should lead to a high infection risk according to Neubauer and Heitmann (2011), the variation between the individual soil samples was very high and there was no correlation between the density of microsclerotia in the soil samples and their particular distances to the next diseased tree. The number of infected trees in a radius of 4 m around the soil sample positions did not correlate with the respective microsclerotia concentrations as well (data not shown). Khan et al. (2000) reported the correlation between inoculum density in the soil and severity and incidence of root discolouration on horseradish, respectively, in greenhouse and microplot experiments. However, they found no correlation in commercial production fields. The authors hence suggested that factors such as temperature, moisture, physiology of individual plants or pathogen strains influenced the inoculum density-disease relationships in the field and concluded that disease-forecast systems were unreliable if these factors could not be controlled. Discrepancies between spatial patterns of microsclerotia in the soil and diseased plants in fields of cauliflower were also shown by Xiao et al. (1997). In spite of the aggregated patterns of microsclerotia, 7 out of 12 sites had shown a random distribution of wilt incidence, which was explained by the authors with very high densities of microsclerotia in almost all samples.

The inconsistency between patterns of microsclerotia concentrations in the soil and distribution of diseased maple trees indicated that *Verticillium* was established in

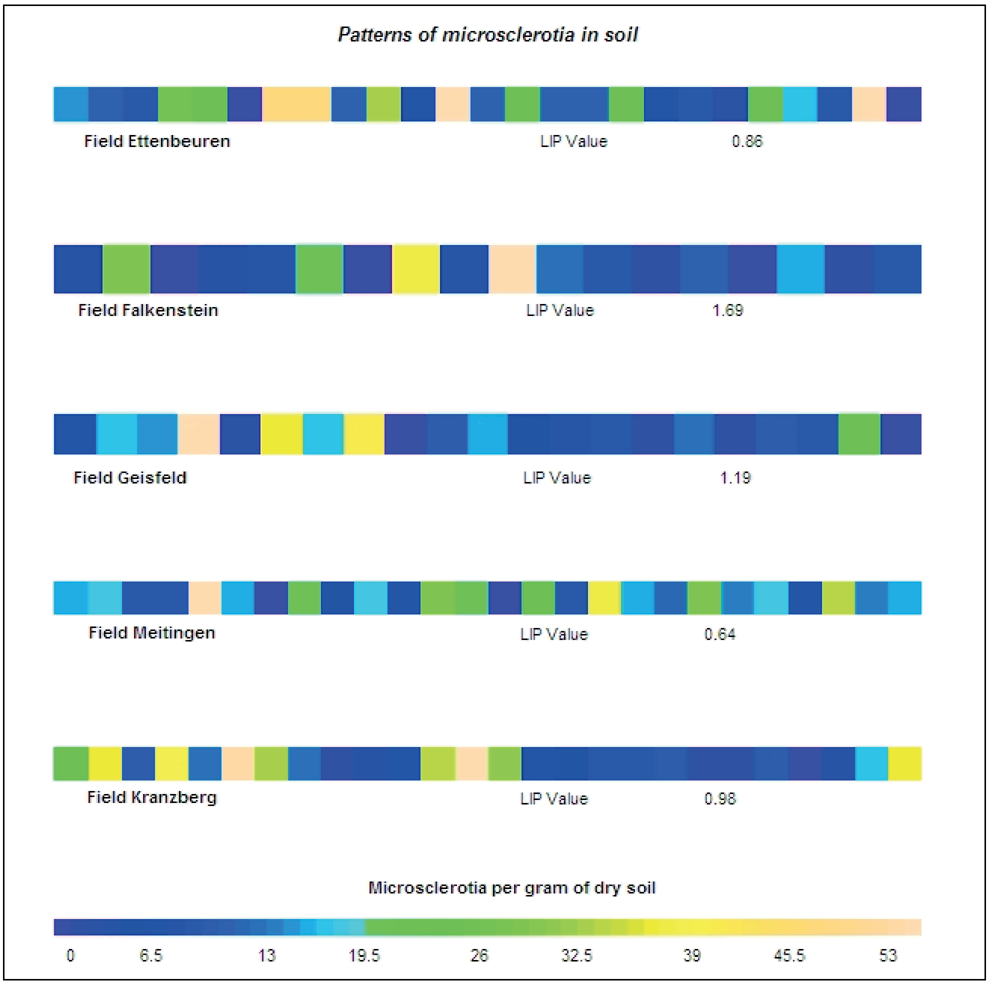

Fig. 3. **Spatial patterns of microsclerotia concentrations along the transects in the sampled plots.** *Verbreitung der Mikrosklerotien entlang der Transekte auf den Probeflächen.*

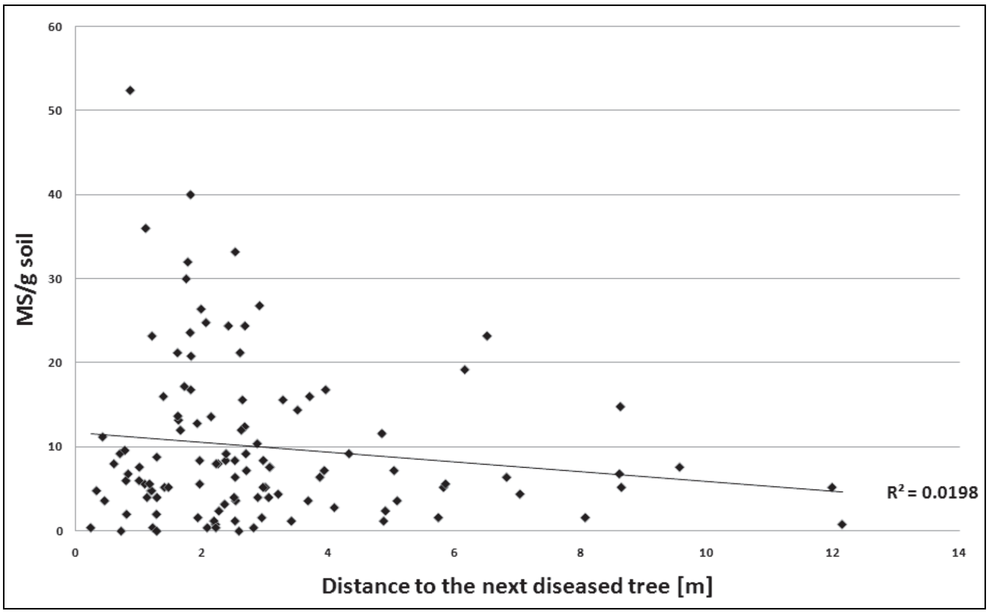

Fig. 4. **Relationship of microsclerotia concentrations to the distance to the next diseased tree.** *Verhältnis zwischen der Konzentration der Mikrosklerotien und ihrem Abstand zum nächsten geschädigten Baum.*

the plots independently from diseased or healthy planted seedlings. One possible explanation is the wide host range of *Verticillium*. Among the plant species forming the surrounding or ground vegetation in maple-dominated forests might be adequate hosts for *Verticillium* as for example *Urtica dioica* or *Rubus allegheniesis* (Pegg and Brady, 2002). Additionally, even though weeds are commonly considered non-hosts for *Verticillium* (Pegg and Brady, 2002), it was demonstrated by various authors that the fungus lives in the vascular system of many weeds with-out causing any symptoms (SKADOW, 1969; EVANS, 1971; VARGAS-MACHUCA et al., 1987). These plants serve as sources of *Verticillium* and contribute to its local accumulation as well as to its distribution by seed transmission (VALLAD et al., 2005). In this study, four out of five plots were located next to grassland. Dissemination of *Verticillium* propagules through anemochorous seeds from graminaceous plants could hence be possible. Moreover, we observed that ground vegetation was less dense in the two plots showing aggregated patterns of microsclerotia concentrations than in the other plots with random distribution of propagules in the soil samples. NAGTZAAM et al. (1997) reported a pathozone width of 0,1 to 0,3 mm, that is, the distance of a microsclerotium to a root must be in this width for a germination and successful infection. Accumulation of microsclerotia should therefore occur in small located patches where the rare host plants grow.

These results suggest that *Verticillium dahliae* is a common fungus in forest ecosystems. However, the more or less simultaneous and accumulative appearance of *Verticillium* infections in several maple populations is striking. The counting of year rings of five infested maple trees from the plot in Kranzberg Forest indicates that the very hot and dry summer 2003 is responsible for the sudden disease of many maple trees, even on soils with a normally very good water supply. This is consistent with TALBOYS and BENNETT (1969) who reported that climate conditions as warm and dry summers favour the disease. The occurrence of weather extremes such as droughts or storms is predicted to increase during the next decades because of the global warming (IPCC, 2007). On this background, the development of maple stockings, especially on suboptimal soils should be intensely observed for new disease incidences.

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