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Research note

Prochloraz tolerance of *Pyrenophora teres* population in Finland

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Barley leaves infected with *Pyrenophora teres* Drechs. f. *teres* were collected from farmers' fields and an experimental field for evaluation of efficacy of fungicides at MTT Agrifood Research Finland (MTT), in 2003. The aim was to test the efficacy of prochloraz to inhibit *in vitro* growth of *P. teres*. Potato dextrose agar (PDA) dishes amended with 0.1 and 1.0 μg ml^{-1} prochloraz were used for testing 364 isolates of *P. teres* based on prelimenary experiment. Isolates from MTT's experimental field were growing slower on fungicide-amended media than isolates from farmers' fields. The overall mean inhibition of radial growth was 63 and 86% on media amended with 0.1 and on 1.0 μg ml^{-1} prochloraz, respectively. Isolates of different origin differed significantly on growth on fungicide-amended media. The isolates capable of growing on increased concentrations of prochloraz were most commonly isolated from fields, where prochloraz was sprayed during the growing season. Within MTT's experimental field no effect of fungicide application during the growing season was observed on growth of isolates *in vitro*. Data from this survey was insufficient for making further conclusions regarding the effect of agricultural practices on selection of fungicide tolerant *P. teres* isolates. Fungicides with different types of mode of action are recommended for use together with prochloraz against the net blotch pathogen in Finland. These results are preliminary.

Key words: barley, *Drechslera teres*, fungicide tolerance, population diversity, sterol-biosynthesis inhibitor, net blotch

Introduction

Prochloraz (1-{*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]carbamoyl}-imidazole; trade names in Finland: Sportak and Prelude) has a wide spectrum of activity and thus can be used on several crops and against several fungal pathogens (Prochloraz: technical information, Hoechst Schering AgrEvo GmbH, Berlin, Germany, 1995). Prochloraz is the most common active ingredient among fungicides used in cereals in Finland (Savela and Hynninen 2004, Plant Production Inspection Centre 2005). It is approved for use against leaf blotch pathogens

Manuscript received September 2005

before sowing as a seed coating and during the growing season as a foliar application, and on winter cereals against pink snow mould before snowfall. In addition, foliar application during the growing season can be split into two separate spraying times with half the amount of active ingredient, resulting in a maximum of four application times for one field during a growing season. To avoid the establishment of fungicide tolerance fungal genotypes, the same fungicide is not recommended for prolonged use on the same crop. Prochloraz belongs to the sterol 14α-demethylation inhibitors (DMIs) and more precisely to the imidazole class. DMIs inhibit the cytochrome P450 dependent oxidative demethylation of eburicol in filamentous fungi as part of the ergosterol biosynthesis pathway (Steffens et al. 1996). Resistance to prochloraz is controlled by a single major gene, and it was acquired after a decade of intensive prochloraz treatments against cereal eyespot pathogen, *Tapesia acuformis* (Dyer et al. 2000). Prochloraz and imazalil, which belong to the same imidazole class of DMIs are the active ingredients for half of the chemical products approved for seed coating of barley in Finland (Plant Protection Inspection Centre 2005). Prochloraz alone is the leading fungicide used for cereals in Finland according to the sales of fungicides (Savela and Hynninen 2004).

Pyrenophora teres Drechs. f *teres* Smedeg. is the cause of net blotch of barley, the most important barley disease in Finland. The average net blotch infection rate between years in Finland depends on variety, but reaches 40% on susceptible varieties without fungicide application during the growing season (Kangas et al. 2004). Fungicide treatment during the growing season results in a significant improvement on yield of susceptible spring barley varieties (Kangas et al. 2005). The risk of losing the efficiency of a fungicide against certain pathogens is greatly increased if the same active ingredient is used repeatedly year after year. Therefore the objective of this study was to test the efficiency of prochloraz to inhibit growth of *P. teres in vitro*, and to find possible differences in prochloraz tolerance among field populations of the net blotch pathogen of barley in Finland. This was a preliminary study.

Material and methods

Sampling of *Pyrenophora teres* populations

Leaf samples were collected randomly during the late growing season after milk ripening stage of spring barley in the Lounais-Häme region near MTT Agrifood Research Finland (MTT), Jokioinen, in 2003. Samples were collected from three farmers' fields (sampling area near two hectares) and a MTT's experimental field (three 16 m^2 plots in four replications), where several fungicides were sprayed during the growing season. Details of barley varieties and fungicide applications during the growing season are given in Table 1. Samples from the same field were considered to represent one population. Single-conidial isolates were established according to McDonald (1967), and kept at –70°C until needed. Isolates were grown on 2.5% V8 medium under near ultra violet light at 18°C, using a 12h light period for two weeks prior to the experiment.

Fungicide tolerance assay

Prochloraz (trademark: Sportak 45 EC (prochloraz 450 g l^{-1}), Bayer Crop Science, Germany) was obtained from MTT Agrifood Research Finland, Evaluation and testing of Fungicides. A radial growth assay was used to determine the tolerance against prochloraz. In preliminary experiment, different dilutions of prochloraz in two culture media (2.5% V8 and potato dextrose agar (PDA)) were used to determine a suitable growing media and dilution for testing of the population samples. Initial dilutions used were: 0.1, 0.5, 2.5, 12.5 and 62.5 µg ml^{-1}. Fungicide was added into cooled liquid media prior to pouring into Petri dishes. Medium without fungicide was used as a control since all the dilutions were made in sterile water. A 7 mm diameter mycelium plug was taken from an actively growing culture of *P. teres* for each type of medium and then placed in the centre of a Petri dish

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Table 1. Characteristics of fields and plots, where barley leaves infected with *Pyrenophora teres* were collected in 2003.

Characteristics	Farmers' field			MTT's experimental field[1)]
	1	2	3	
Barley variety	Scarlett	Annabell	Rolfi	Rolfi
Active ingredient of fungicide	Prochloraz	Prochloraz	Azoxystrobin	a) Azoxystrobin + Propiconazole, Fenpropidin mixture b) Prochloraz c) Prochloraz
Fungicide rate (l ha^{-1} of product)	0.5	0.6	Unknown	a) 0.4 + 0.4 b) 0.5 c) 1.0
Number of single conidial isolates	52	51	59	a) 54 b) 62 c) 86

[1)] Samples were collected separately from fungicide treated plots, taken into account in the statistical analysis.

containing prochloraz-amended medium. Three replicates were used. Cultures were incubated under near ultra violet light at 18°C using the 12 h light period until the fungus in the control Petri dishes reached the edges of the dishes. The diameter of radial growth of the fungus, including the inoculum plug, was measured (mm) from all Petri dishes on the same day. Test isolates did not grow on concentrations of 2.5 µg ml^{-1} prochloraz medium or stronger. Therefore concentrations of 0.1 µg ml^{-1} and 1.0 µg ml^{-1} prochloraz were chosen as treatments for testing the field populations of *P. teres*. PDA was chosen as test medium because growth of the fungus was more difficult to measure visually on darker V8 media. *P. teres* isolates were tested in two successive experiments, both having two replicates per isolate for each treatment. Control was included in all experiments with two replicates for each isolate. Cultures were incubated for 7 days prior to measuring colony growth as described above.

Data analysis

The radius of the inoculum plug (7 mm) was substracted from the growth measures before statistical analysis. Radial growth of each isolate on fungicide-amended media was calculated as a proportion of the growth on the control medium. This corrects for differences in growth rates between isolates (Peever and Milgroom 1993). Analyses of variances were performed using PROC GLM in SAS® Proprietary Software Release 8.2 (SAS Institute Inc., Cary, NC, USA). The residuals of proportional growth were close to a normal distribution on data from 0.1 µg ml^{-1} prochloraz medium, whereas the residual distribution for data from the 1.0 µg ml^{-1} prochloraz medium was wider than but close to the normal distribution. Transformations on data were considered unnecessary. Firstly, we tested the hypothesis that isolates of *P. teres* from each plot within MTT's experimental field were homogenous. Secondly, we hypothesised that there were no differences between populations in proportional growth on fungicide-amended medium. Three sources of variation were included in analysis: repetition of experiment, field (or plots within MTT's field) and isolate (within field/plot variation).

Results

Isolates from MTT's experimental field

Growth differed significantly from zero for both concentrations of prochloraz ($P < 0.0001$). The

sampling plot of an isolate did not have marked effect on growth differences. However, an interaction between repetition of experiment and plot was found for both concentrations of prochloraz ($F = 101.48$, $P < 0.0001$ and $F = 25.64$, $P < 0.0001$ on 0.1 and on 1.0 µg ml^{-1} medium, respectively). These significant interactions were mainly due to high variation between the two experiments among growth results for isolates originating from plot b, which were growing weakly on the first experiment. The overall mean radial growth of *P. teres* isolates originating from MTT's experimental field was 7 and 1 mm, while the proportional growth was 0.18 and 0.03 on 0.1 µg ml^{-1} prochloraz medium and on 1.0 µg ml^{-1} medium, respectively. The distribution of isolates (sum of two experiments) growing on fungicide-amended media is shown in Figure 1 and 2 (MTT's field).

Combined data

Data from both the experimental and farmer's fields were combined to test whether the isolates from different fields differed in their ability to grow on fungicide-amended media. Two of the farmers' fields and two plots within the MTT's experimental field had prochloraz applied during the growing season, whereas the third farmer's field and a plot of the MTT's experimental field had an azoxystrobin-based fungicide applied (Table 1). The effect of field of *P. teres* isolates on propor-

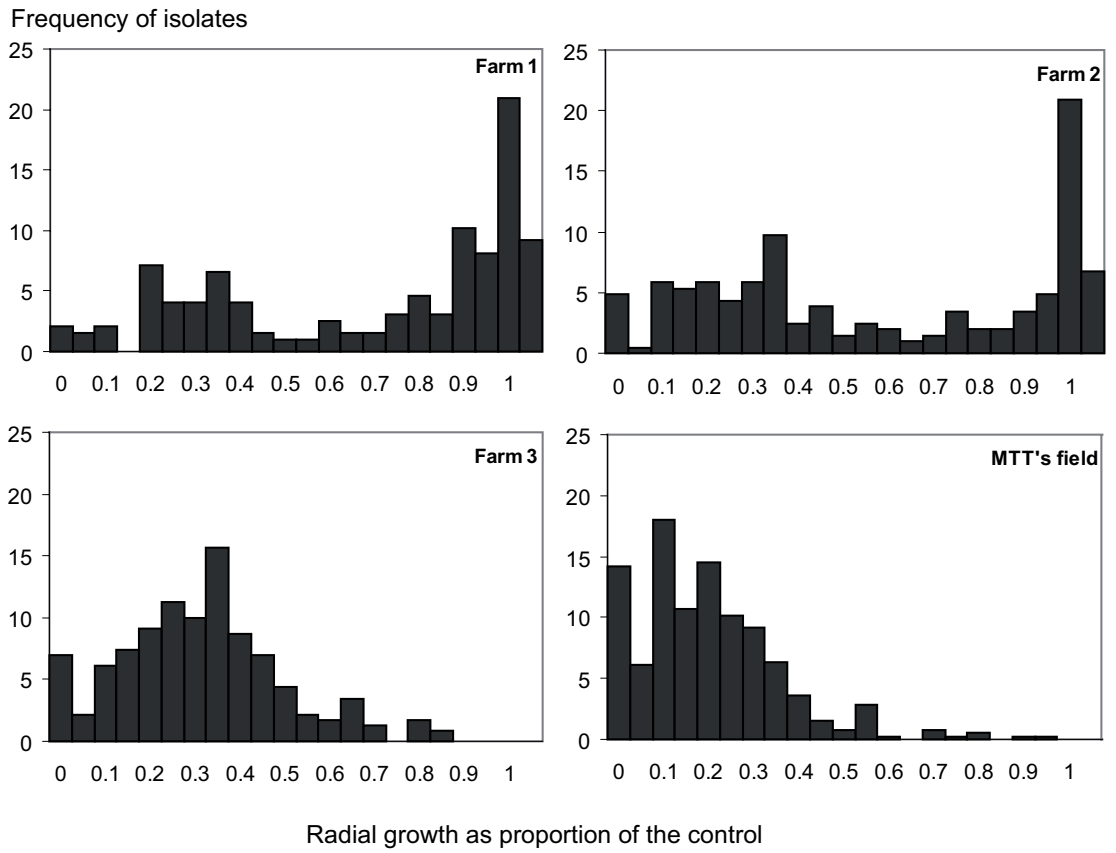

Fig. 1. Distribution of radial growth of *P. teres* isolates (frequency) growing on 0.1 µg ml^{-1} prochloraz-amended medium as proportional growth of the control media.

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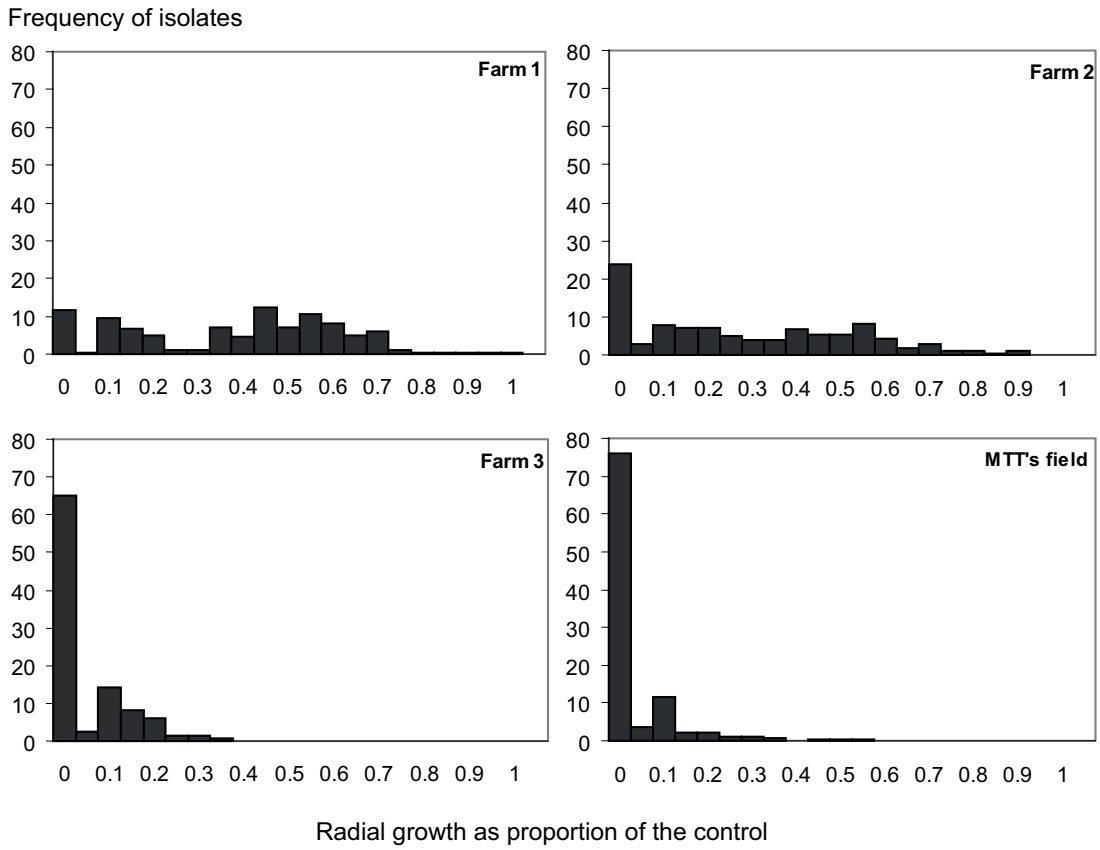

Fig. 2. Distribution of radial growth of *P. teres* isolates (frequency) growing on 1 µg ml^{-1} prochloraz-amended medium as proportional growth of the control media.

tional growth was significant on both media ($F = 212$, $P < 0.0001$ and $F = 267$, $P < 0.0001$ on 0.1 and on 1.0 µg ml^{-1} prochloraz medium, respectively). Most of the variation was between fields, namely 38.0 and 43.5% on 0.1 and 1.0 µg ml^{-1} prochloraz medium, respectively. Less than 1% of the total variation was due to different fungicide treatments during the growing season (i.e. the effect of fields with different fungicide regimes). No significant effects were found among plots within MTT's experimental field. In addition, difference of normalised growth was high between farmers' fields and MTT's experimental field. The overall mean radial growth of isolates originating from farmers' fields was 19 mm and 8 mm, and the proportional growth 0.50 and 0.21 on 0.1 and 1.0 µg ml^{-1} prochloraz medium, respectively. The growth of isolates originating form MTT's experimental field was approximately one third of that. Isolates originating from farmers' field 1 and 2 had the highest growth rates on fungicide-amended media (Figure 1 and 2).

Efficiency of prochloraz

The percentage of all isolates, in which growth was inhibited less than 50% was 27.1% on 0.1 µg

ml^{-1} prochloraz medium and 10.8% on 1.0 μg ml^{-1} medium. The percentage of all isolates growing as well or better on 0.1 μg ml^{-1} prochloraz medium compared with the control medium was 3%. Most of these isolates were collected from fields that had been sprayed with prochloraz during the growing season (Farms 1, 2 in Figure 1). Only one isolate (from Farm 1) had similar growth rates on 1.0 μg ml^{-1} prochloraz medium as compared to the control medium. In contrast, the number of all isolates with less than 5% of the growth of the control when cultured on the 0.1 μg ml^{-1} prochloraz medium was 11.6% and 53.5% on 1.0 μg ml^{-1} medium. These isolates mostly originated from farmer's field 3 and from MTT's experimental field (Figure 1 and 2).

Discussion

The radial growth assay experiments were performed during the mid-winter in growth chambers in laboratory, where the effect of environmental factors was minimised. Still results demonstrated that experiment had an effect on growth of isolates. The variation in growth was marked within isolates and among experiments. In spite of the effect of experiment, results indicated that field origin of *P. teres* isolates had an effect on radial growth *in vitro*. The EC_{50} value for *P. teres* was 0.026 μg ml^{-1} of prochloraz in Sweden in 1982 before the fungicide Sportak 45 EC was registered for use in there (Olvång 1988). Concentrations of 1 μg ml^{-1} of prochloraz resulted in 90% growth inhibition of *P. teres* isolates from Sweden (Olvång 1988). In this study the overall mean growth inhibition of *P. teres* isolates was 86% (79% for farmer's fields, and 69% for Farms 1 and 2) with the same concentration. Since earlier results from Finland are lacking, we cannot conclude that the effectiveness of prochloraz has changed during the years. However, *P. teres* isolates originating from farmers' fields, in which prochloraz was sprayed during the growing season had increased growth on fungicide-amended media. It is possible that isolates from farmers' fields have been under stronger selection pressure.

Prochloraz can be used in the same field several times, while the net blotch pathogen population can be maintained in the field for several years. *P. teres* survives over the winter in infected stubble (Jordan 1981) and Finnish *P. teres* populations seem to be genetically differentiated between fields (Serenius et al. 2005). At least one of the farmers' fields had barley cultivation before the study year, while another field was not ploughed before barley was sown. Continuous barley cultivation and reduced tillage are factors that can increase the net blotch infection risk on barley (Jordan 1981). However, this survey was not established to test the effect of fungicide application during the growing season or the effect of other agricultural factors, such as variety or ploughing on natural selection of fungicide tolerant *P. teres* isolates. Therefore a more precise experimental design and a sufficient number of samples are needed to study factors affecting the selection of fungicide tolerant *P. teres* isolates. The current study did not indicate significant differences between plots within MTT's experimental field. Our earlier studies have identified little or no differentiation in *P. teres* populations within fields based on AFLP markers (unpublished data), but high differentiation between fields in Finland (Serenius et al. 2005). Short distance spore dispersal may help to distribute genotypes of *P. teres* resulting in more uniform within field populations with similar fungicide sensitivities. While the potential for spore dispersal over long distances is unclear, it may be limited, which may result in greater differentiation between fields. However, seed-borne infection with *P. teres* may help to move novel genotypes of *P. teres* over long distances.

Olvång (1988) found high variation in sensitivity of *P. teres* isolates to prochloraz, and the population was divided into two groups: sensitive and less sensitive. We identified a marked effect of field of isolates on growth on prochloraz-amended media. In the two farmers' fields, in which prochloraz spraying took place, the mean growth rates on prochloraz-amended media were similar and were higher compared with other *P. teres* populations.

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The high variation between experiments within isolates originating from MTT's experimental field precluded the detection of differences among isolates taken from plots with and without the application of prochloraz.

Implications

To maintain the efficiency of fungicides, evaluation of long-term effectiveness of fungicides on cereal plant pathogens is important. Fungicides with different modes of action should be used on cereals according to official recommendations. Sales of fungicides in Finland (Savela and Hynninen 2004, Plant Production Inspection Centre 2005) show that fungicides with two different active ingredients and modes of action are beginning to replace fungicides with only one active ingredient, which is a positive change. However, different active ingredients may still have the same type of mode of action, *e.g.* fungicides belonging to imadazole class of DMIs (imazalil and prochloraz). In addition, the occurrence of cross-resistance has been observed in *P. teres* among imazalil-propiconazole and fenarimol-triadimenol, which are all sterol-inhibiting fungicides, but more importantly belong to different chemical groups among the DMIs (Peever and Milgroom 1993). Under prolonged use of the same type of fungicides, less sensitive isolates will survive in the pathogen population residing in a particular field (Olvång 1988). Sexual reproduction can combine genes effectively and potentially produce more tolerant isolates. This risk must be taken into account since sexual reproduction of *P. teres* is possible in Finland (Serenius et al. 2005). However, to fully understand the population biology of the net blotch pathogen and the potential for development and spread of fungicide tolerant isolates more research is needed.

Acknowledgements. Tarja Hovivuori is thanked for her technical assistance. The authors want to acknowledge the help and co-operation of people that work in the Evaluation and testing of plant production products at MTT Agrifood Research Finland. This work was supported by grants from the Finnish Cultural Foundation and Finnish Ministry of Agriculture and Forestry.

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SELOSTUS

Ohranverkkolaikkutaudin aiheuttajan prokloratsin kestävyys Suomessa

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Ohranverkkolaikku on tärkein taudinaiheuttaja suomalaisessa ohranviljelyssä, ja se aiheuttaa aroilla ohralajikkeilla keskimäärin 40 % tautisuuden ja merkittäviä satotappioita. Tautia torjutaan siemenpeittauksella tai kasvustoruiskutuksilla kasvukauden aikana. Prokloratsi (kauppavalmisteet Sportak ja Predule) on yleinen ohran kasvinsuojeluaine. Suomessa myydyistä peittausaineista puolet sisältää vaikuttavana aineena joko prokloratsia tai imatsaliilia tai näiden yhdistelmiä. Molemmat aineet kuuluvat samaan kemialliseen ryhmään ja vaikuttavat samalla tavalla. Tämän tutkimuksen tarkoituksena oli selvittää prokloratsia kestävien ohranverkkolaikkua aiheuttavien tautikantojen esiintymistä lounaishämäläisillä pelloilla.

Näytteitä kerättiin yhteensä 364 kpl vuonna 2003 kolmelta viljelijän pellolta ja yhdeltä MTT:n (Maa- ja elintarviketalouden tutkimuskeskus) koealalta. Viljelijöiden pelloilta kerätyt tautikannat, jotka olivat saaneet Sportak-käsittelyn kasvukauden aikana, kasvoivat laboratoriotesteissä paremmin kasvitautiainetta sisältävillä kasvatusmaljoilla kuin koealalta kerätyt tautikannat. Viljelijöiden pelloilta peräisin olevat tautikannat kasvoivat parhaiten sekä laimeammalla että vahvemmalla testialustalla verrattuna tautikantoihin, joilla oli käytetty muita kasvinsuojeluaineita. Vastaavaa testiä ei ole tehty aiemmin, joten ei voida osoittaa tautiaineen kestävyyden muuttuneen. Tulokset osoittavat, että tautikantojen kasvinsuojeluaineen kestävyys voi vaihdella eri pelloilla. Tämä alustava aineisto oli kuitenkin riittämätön yleistettävien johtopäätösten tekoon.

Aineiden tehoa kannattaa seurata. Suositeltavinta olisi käyttää aineseoksia ohranverkkolaikun torjunnassa ja välttää samalla tavalla vaikuttavien kemiallisten kasvinsuojeluaineiden pitkäaikaista käyttöä samalla peltolohkolla. Tautiaineiden tehon turvaamiseksi tautia pitäisi pyrkiä vähentämään myös muilla keinoilla, kuten viljelykierrolla, kasvijätteiden muokkauksella ja taudinkestävien ohralajikkeiden viljelyllä. Tämä alustava tutkimus osoitti, että ohranverkkolaikun aiheuttajan prokloratsin kestävyydessä on eroa peltojen välillä.