Diagnostics of phytopathogen infection in agricultural plants as a necessary condition for optimizing current fungicide application technologies

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Existing methods for diagnostics of phytopathogen infection in agricultural plants are analyzed. Advantages and lacks of indirect and direct methods of infection incidence assessment are discussed. An indicator method widely applied for diagnostics of fruit crop infections is considered. The conclusion about necessity of indicating plant infection in the earliest phases of phytopathogen growth is made.

Key words: diagnostics, fungicide application, plant disease

Introduction

The effectiveness of chemical protection of agricultural plants from diseases often depends not only on the fungicide applied, but also on correct terms of its application. The existing ways of determining probable necessity and terms of fungicide application are predominantly based on indirect prediction of plant infection. In the last years, techniques for direct diagnosis of plant phytopathogen infection in the latent phase have been intensively developed.

Methods for determining the necessity and terms of fungicide application include: indirect prediction of plant infection and direct diagnosis of plant infection. Technical schedule, phenological forecast, prognostic model of infection risk periods, visible manifestations of the earliest disease symptoms are list of the indirect prediction methods. Direct diagnosis of plant infection include the direct identification of pathogen in plant tissues (immunofluorescence staining, ELISA and PCR diagnostics) and detection of pathogen metabolic products ergosteroles and microtoxins.

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It is well known that application of fungicides within 2-3 days after spore germination influences strongly pathogen activity (Parry *et al*., 1995; Chmyr and Kolesova, 1996; O'Leardy and Sutton, 1986; Jahn and Burth, 1998; Klink et al., 1998), thus providing very slow development of pathogen resistance to the fungicides.

Diagnostics of plant infection in the latent phase of pathogen development is also important for agricultural plant breeding. Absence of a rapid diagnostic technology impedes selecting pathogen resistant varieties. Appearance of visual infection symptoms serves usually a criterion of variety effectiveness in terms of pathogen resistance (Geshele, 1978; Statler and Parlevliet, 1987; Zhdanov and Sedov, 1991). Thus the mechanism of pathogen plant resistance can be judged a priori from the results of numerous experiments (Geshele, 1978). For instance, in breeding apple scab resistant apple tree types, short incubation period, plentiful and long spore production of *Venturia inaequalis*, a causative agent of the apple scab, are characteristic of susceptible plants (Zhdanov and Sedov, 1991). After inoculation, the display of strong disease lesions takes 12-14 days in susceptible apple tree types and up to 29 days in resistant ones. Long delay in strong disease lesion display is one of the elements of field (or horizontal) resistance of plants to pathogen.

Replacement of preventive fungicidal preparations for curative ones has induced an interest to early diagnostics of plant diseases. Application of currently available fungicides in early phases of phytopathogen growth has a number of advantages. First of all, harmful effect of the infection is prevented at the earliest stage of illness, i.e. before mycelium destroys vegetative cells. Purposeful fungicide application enables to reduce the number of treatments thus elevating cost effectiveness of plant protection itself. Numerous experiments on early application of fungicides on various agricultural plants have confirmed the above statements.

Early diagnostics of plant fungous diseases seems to be vital for biological control of plant infections, since a lot of research testifies that bioagents most effectively suppress fungus pathogens in the phase of spore germination and at the beginning of mycelium growth (Hofstein et al., 1996; Fokkema, 1996).

It should be noted that early diagnostics is complicated by the fact that latent phase of pathogen growth lasts from 5 up to 30 and more days depending on pathogen host interrelationship and weather conditions. Some pathogens such as *Monilia* spp. have as long latent phase as 100 days and more. During this very period mycelium of the fungus destroys vegetative cells and causes their die-off. In this case, appearance of visible disease signs serves an overdue signal for fungicide application. Critical periods, i.e. periods when cumulative

effect of various factors (weather, humidity, phase of pathogen growth, etc.), promote plant infection, occur, as a rule, repeatedly during one season.

The paper presented is aimed at summarizing literature and own data on the methods of early diagnostics of phytopathogen infection of crops and revealing the most prospective of them. Early diagnostics of phytopathogen infection will allow for correct forecast of plant disease development, for determining necessity and terms of fungicide application in order to reduce the cost of protection.

Indirect methods of plant infection assessment within an agrocenosis

The history of fungicide application in world agriculture begins from the 1950s. Since the 1960-1970s various technologies of fungicide application have been tested. In those years, and quite often nowadays, a scheduled application of fungicides is used. The scheduled application implies treatments in certain intervals, usually 7-15 days, depending on the time of protective activity of the agent used. However, this way of fungicide application does not practically consider such important factors as plant susceptibility and pathogen presence. As a result, scheduled application of fungicides may make no effect due to the absence of the threat of infection. Besides, fungicide application does not give desirable effect in the years of outbreaks, when the application terms do not coincide with the critical periods of phytopathogen impact. Nevertheless, the scheduled application is suitable due to low cost of the preparations used.

Alongside with the scheduled fungicide application, the so-called phenological forecast is a common practice. The phenological forecast implies fungicide application in a definite phase of plant growth. In this case there should be a synchronism between ontogenesis of pathogen and plant-host development. Our long-term observations and the literature data indicate that such synchronism is frequently broken. For example, unusually early spring warming induces pathogen development surpassing considerably plant awakening, due to which phenological forecast often gives an overdue signal for fungicide application making the latter useless.

Quite often visible disease symptoms serve an indication of the forthcoming infection outbreak, and fungicide application is usually recommended at 5 % disease incidence (Sanin et al., 1997a, 1997b). However, the latent period of pathogen development actually comes to the end by this moment and, hence, harmful effect is strongly displayed, and the signal to apply fungicide turns to be too late.

In the last years prognostic models have been used to define optimum terms of fungicide application. Construction of such models is based on the effect of weather factors on illness development. Weather conditions, especially temperature and humidity, influence essentially infection processes and pathogen spore germination, incubation period, disease symptom display, spore formation and other infectious factors including phytopathogen distribution (Stepanov and Chymakov, 1972). The knowledge of these factors is an obligatory condition for prognostic model construction.

Theoretical bases of phytopathologic forecast were established in the 1950s, when wide application of fungicides began. Interest to the forecast has sharply increased by the end of 1960 - beginning of 1970, which is indicated by numerous scientific articles and practical recommendations concerning outbreaks of some plant infections as well as by publications of summarizing and theoretical character (Stepanov and Chymakov, 1972; Polyakov and Chymakov, 1968; Zsadox, 1970).

By the end of the 1980s beginning of the 1990s the forecast has attracted even more attention in connection with the replacement of preventive fungicides for the so-called curative preparations possessing a systemic mechanism of action. Computerization of forecasting process and arrangement of special agricultural-weather loggers allow for monitoring parameters, such as air temperature, leaf moisture degree and duration, etc., necessary for predicting plant infection upsurges.

Some biological parameters, such as plant-host development, pathogen spore size (Starodub et al., 1994; MacHardy, 1994; Trapman, 1994), measured with various spore detectors (MacHardy, 1994; Evsyukov and Sokolov, 1994; Evsyukov et al., 1997; Sadkovsky and Sokolov, 1997) have been considered in recent prognostic models. Such prognostic models are capable of determining the terms and duration of plant infection upsurges. Attempts to predict infection severity classifying it as weak, moderate and strong are sometimes also possible. In many countries of the world (USA, England, Germany, etc.), prognostic models have been involved in national programs on forecasting and control of diseases in economically significant crops for more than 20 years (Sanin, 1997; Dolz and Galli, 1994).

However, predicting models use some indirect parameters subjected to different changes often not predictable even within one day. For example, daily average air temperature, phytopathogen and plant-host development intensity are not identical in time even within one agro-ecological region. These parameters depend on numerous factors, such as structure and micro relief of areas under crops and perennial plants, crop cultivation technologies, microclimate, etc. (Sanin, 1997). In this connection the use of prognostic

models for deciding the necessity and terms of fungicide application may have a significant share of risk.

There are numerous reports of foreign and Russian researchers that the models quite often predict much more plant infection upsurges than it might actually be (Trapman, 1994; O'Leardy et al., 1987; Sutton and Nardocei, 1983; Triloff, 1994; Fedorova et al., 1994). For example, testing of an Austrian agricultural-weather logger predicted 16 critical periods of apple tree scab infection, however, only 4 treatments could actually be enough (Fedorova et al., 1994). It is not infrequently when models do not signal about danger whereas severe infection is actually observed (Trapman, 1994; Triloff, 1994). This seems to be one of the reasons why agro-weather loggers have not had wide practical application until now. Although in some European countries (Germany, Austria, etc.) a network of such loggers has worked for about 20 years to inform the farmers about the terms of effective fungicide application. Farmers usually prefer to spend money for buying fungicides rather than to have a risk by using model predictions, first of all worrying about stably high crop, and not about preservation of the environment and product safety.

Direct plant infection assessment

In the last years methods of detecting and identifying phytopathogens in early growth phases on the base of the analysis of toxins produced by them have been intensively developed. These methods have found the widest application in diagnostics of cereal infections caused by fungi of the genus *Fusarium* synthesizing fusarium toxins, such as desoxynivalenol (DON, or vomitoxin), its acetyl derivatives, T-2-toxin, zearalenon, etc.

Shipilova et al. (1994) recommended DON content assessment for diagnostics of *Fusarium* induced infection of cereal heads and kernels as the most correct method. Experiments performed in conditions of high humidity of a season of 1993 (Leningrad Region, Russia), when in wheat grains with latent *F. avenaceum* – caused infection vomitoxin content had reached threshold values by the time of wheat maturation showed that DON can be accumulated in infected kernels even if no visible signs of infection are evident.

Some authors have shown (Scott et al., 1987; Mills et al., 1988; Thrane, 1989; Leonov, 1991; L'vova et al., 1994; Tejada-Simon, 1995) that toxin-producing ability of *Fusarium* species can be used for species specific identification and detection of the pathogen in early phases of its growth.

Liu and Wang (1990) used DON content assessment as a criterion of *Fusarium* infection. A system of *in vitro* screening for the assessment of triticale resistance to *Fusarium* infection was developed by Maier and Oettler

(1993). It was found out that in the callus of resistant triticale varieties DON content is increased in comparison with susceptible varieties. This method is assumed to be applicable for selecting resistant genotypes of other crops in laboratory conditions. However Bruins et al. (1993) did not reveal a correlation between DON content in the callus and field resistance of crops to *Fusarium* infection.

Alongside with chemical methods of toxin detection, ELISA implying the detection of antigens (antibodies) accumulated in vegetative tissues of plants infected with the fungus is also used (Clear et al., 1996). In the authors' opinion, the method can find application in the selection of resistant and non-contaminatable varieties of cereals, for studying a correlation between kernel infection and toxin accumulation, which would be helpful for assessing efficiency of methods to control phytopathogens. ELISA was used also for estimating DON content in barley and oats.

Immunofluorescence direct microscopy is used for detecting pathogenic fungi in soils (Dewey et al., 1984; Koziakov, 1987). Immunofluorescent coloring is reliably good not only for identifying micromycetes in any environment, but also for monitoring the development of each micromycete structure. Strunnikova et al., (1998) and Shakhnazarova et al. (1999) successfully used the method of membrane filters and immunofluorescence for studying *Fusarium culmorum* distribution. The method of membrane filters (Adams, 1967) in combination with immunofluorescent coloring appeared to be quite suitable for long-term monitoring the development of all morphological structures in *F. culmorum* population. Fluorescent microscopy was used for studying the resistance of spring barley to *Puccinia glumarum*, a yellow rust causative agent, in early phases of the pathogen growth (Munnich et al., 1998).

Among new generation methods, PCR deserves some attention. The method is based on DNA amplification and use of known nucleotide sequences as markers for detecting pathogens in plant tissues. PCR analysis for identification of some fungus pathogens, including *F. graminearum* (Quiller and Scrift, 1993), *F. poae* (Parry et al., 1996), *Pseudocercosporella herpotrichoides* (Popard et al., 1993), *Gaeumannomyces graminis* (Schesser et al., 1991), *Microdochium nivale* and *Rhizoctonia cerealis* (Nicholson and Parry, 1996) in cereals was developed. Initially the method was used for identification of fungi grown on an agar nutrient medium, and then it was found applicable for detecting pathogens in the tissues of infected plants.

PCR assay seems to be perspective for differential diagnostics of plant diseases. For instance, differentiation of symptoms of infections caused by *R. cerealis* and *P. herpotrichoides* is especially complicated in early phases of

cereals growth (Goulds and Polley, 1990). Using PCR, the authors (Nicholson and Parry, 1996) could identify *R. cerealis* and *P. herpotrichoides* which parasitize on one and the same plant in early growth phase (phase 37). Good correlation between visual disease symptoms and the results of PCR assay of matured plants (phase 75) was found out. PCR assay appeared also helpful in identifying confusion species of *Monilia* (Hughes et al., 2000). There is reason to believe that PCR will find application in a number of areas, including forecasting of infection outbreaks at early stages and studying interrelations between pathogens inhabiting one and the same plant for better understanding the contribution of each pathogen to the infection caused crop losses.

Current methods most intensively developed abroad, are frequently inaccessible for practical plant protection service (Sanin, 1997). Being complicated in use, labor-consuming and intended for application in specialized laboratories, these methods can hardly be applicable for rapid decision about the necessity and terms of fungicides to control agricultural plant diseases. Besides, high sensitivity and specificity of the methods with regard to infection causative agent, for example, *Septoria nodorum*, make difficult getting the information about the quantity of infectious material.

An indicator method seems to be optimum for practical application. The method is easy in use, accessible for farmers and effective in detecting a pathogen in plant tissues even in the phase of spore germination (Chmyr and Kolesova, 1997).

The indicator method involves application of an indicator solution containing a chemical dye on the surface of leaves, stems or fruit, which have no external symptoms of illness. If the plant is damaged and latent infection takes place, the indicator paints the pathogen brightly making it well visible like pictures developed on a photographic paper. The terms and degree of actual infection can be judged from the number and size of the displayed foci and the number of spores germinated. Based on this information, one can decide about the terms and necessity of fungicide application even 5-7 days prior the appearance of visible infection symptoms. It is no less important that the farmer can make testing of the pathogen *in situ,* i.e. immediately in field conditions. The method has been successfully tested for identifying apple and pear scab, grapes mildew, *Phytophthora infestans* on potato and tomatoes, *Puccinia* spp. and *Septoria* spp. on cereals (Chmyr and Kolesova, 1996; Kolesova and Chmyr, 1996; Chmir and Kolesova, 1997).

In large-scale industrial testing, application of current chemical fungicides in early pathogen growth phases (5-7 days before the display of visible symptoms) detected using an indicator, reduced the number of

treatments no less than 2-fold, the efficacy being increased (Chmyr and Kolesova, 1998; Kolesova and Chmyr, 1998).

Besides, the indicator method makes it possible to determine fungicide efficiency from the status of pathogen mycelium (alive or dead) in the incubation phase even in 1-2 days after treatment. The method appears to be effective for evaluating pathogen mycelium status depending on hydrothermal conditions, which is helpful for avoiding unnecessary fungicide application in case of natural mycelium destruction in the incubation phase.

Nevertheless, despite clear advantages of the indicator method for early diagnostics of agricultural plant diseases, it could hardly cover all the pathogens in the latent growth phase.

At present, the authors of the given paper are carrying out research on adaptation of the designed indicator method for identifying *Fusarium* blight in wheat heads and leaves. 30 various dyes, including standard indicator, have been already tested. Even first results obtained on various plants infected with *F. graminearum* showed that the method is prospective. In the experiments, wheat heads during flowering were infected by immersing into *F. graminearum* spore and macerated mycelium suspension. The indicator showed the presence of fungus in kernel rudiments even in 3 days after inoculation. In the experiments on infected shoots of cereals (winter and spring wheat, barley) the pathogen was detected also on the third day after the inoculation of plant leaves with *F. graminearum* by the same technique. Small pink spots appeared on the leaves apparently indicating the places of mycelium congestion.

The results obtained testify that though standard indicator is capable of detecting *Fusarium* head blight in the early phase of pathogen growth, its sensitivity is still insufficient. At the same time, diseases of fruit crops can be diagnosed in 3-4 h after inoculation. Probably, differences in the results can be explained by the fact that Fusarium fungus hyphae in the initial growth phase are very thin, absorb not enough dye, consequently become visible in a lag period after exposure to the indicator and require detecting under a microscope. American researchers (Pritsch et al., 2000), using molecular-genetic methods, have shown that *F. graminearum* is detectable in plant tissues even in 36-48 h after inoculation. Thus, searching for a rapid method of the detection of *Fusarium* infection in the early phase of pathogen growth, namely before the formation and maturing of kernels in the heads, should be continued.

Conclusion

Numerous studies confirm that forecasting of terms of the most rational fungicide application is an integrated problem, and the role of a set of factors influencing interrelation between the pathogen and plant-host should be considered to resolve it. The forecasting can be made from potential pathogen infection (indirect assessment) and from actual presence of pathogen (direct assessment).

Detection of phytopathogens in plant tissues in early growth phases is of prime importance. Direct assessment of the presence of pathogen on plants with the purpose of prevention and control of plant diseases can be made from indirect pathogen activity indications and from the results of phytosanitary monitoring of fungicide efficacy.

Direct assessment of the presence of phytopathogen in plant tissues in early growth phases could be use of ELISA, PCR assay, imunofluorescence methods and method of analysis of micromycete metabolism products, such as ergosteroles, mycotoxins: DON and its acetyl derivatives, T-2-toxin, zearalenon, etc. Unfortunately, being not easy in use, labor consuming and specified for laboratory application, these methods are not usable for rapid diagnostics of agricultural plant diseases with the purpose of making a decision about necessity and terms of fungicide application.

The indicator method described above seems to be the most prospective for practical use. Efficiency of this method depends in many respects on physiological and biochemical characteristics of the pathogen and on the mechanisms of interaction of the latter with the plant-host.

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