PHYSIOLOGICAL EFFECTS OF STROBILURIN FUNGICIDES ON PLANTS

EFEITOS FISIOLÓGICOS DE FUNGICIDAS DO GRUPO DAS ESTROBILURINAS SOBRE PLANTAS

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SUMMARY

The interference of plant diseases results in significant losses in the productivity and quality of products obtained. This situation is growing and progressing due to plant breeding aimed at increasing productivity, and the increase of agricultural borders, thus providing food that is more susceptible to phytopathogens during all seasons of the year. As a result of this action of microorganisms, man is obligated to take steps to neutralize this negative effect, combining other available techniques with the fungicides. Until recently, the fungicides focused on control of phytopathogens with the sole purpose of reducing inoculum. After the launching of strobilurins, and with the evolution of this group of chemical products, the concept of disease control gained new perspectives, especially when considering the advantages obtained by the action of positive physiological effects on these plants. During the last decade of intense research on the fungicidal properties of strobilurins, the evidences of their direct influence in physiological processes of plants not infected or threatened by pathogens were strengthened. This activity was named “physiological effect”. The physiological effects of pyraclostrobin, a new molecule of strobilurin, were reviewed under several levels of complexity, from the greening effect frequently mentioned and the enhancement of stress factors in field and under controlled conditions, to the influence of hormonal regulation and assimilation of carbon and nitrogen by the plant.

Key words: pyraclostrobin, F500, physiological effects, chemical control
RESUMO

A interferência das doenças de plantas resulta em perdas significativas na produtividade e qualidade dos produtos obtidos. Esta situação torna-se crescente e progressiva em função do melhoramento vegetal visando aumento de produtividade e do aumento das fronteiras agrícolas, disponibilizando, desta forma, alimentos mais suscetíveis para os fitopatógenos durante todas as estações do ano. Como consequência desta ação de microorganismos, o homem se obriga a ações que neutralizem este efeito negativo, associando às demais técnicas disponíveis os fungicidas. Até recentemente, os fungicidas tinham como foco o controle de fitopatógenos visando exclusivamente a redução do inóculo. A partir do lançamento das estrobilurinas, e com a evolução deste grupo de produtos químicos, o conceito de controle ganha novas perspectivas, principalmente considerando as vantagens obtidas pela ação de efeitos fisiológicos positivos deste sobre as plantas. Durante a última década de intensa pesquisa sobre propriedades fungicidas de estrobilurinas, foram intensificadas as evidências sobre influências diretas em processos fisiológicos de plantas não infectadas ou ameaçadas por patógenos. A essa atividade denominou-se “efeito fisiológico”. Os efeitos fisiológicos do pyraclostrobin, uma nova molécula de estrobilurina, foram revisados sob diversos níveis de complexidade, desde o efeito verdejante (greening) frequentemente mencionado e melhoria de fatores estressantes em campo e sob condições controladas, até influências de regulação hormonal e assimilação de carbono e nitrogênio pela planta.

Palavras-chave: pyraclostrobin, F500, efeitos fisiológicos, controle químico

1. Introduction

Agricultural practices, observed through the physiological aspect, aim at maximizing the photosynthetic efficiency of cultures and directing photoassimilates toward the formation of grains and other yield factors, instead of other unproductive energy consumptions. The infections caused by fungi impair the efficiency of the cultures, reducing the tissue area for photosynthetic activity and inhibiting the translocation of assimilates, from their source of production up to the areas of growth and deposition of yield material (grains, fruits, etc.). The diseases also deviate the photoassimilates toward unproductive consumptions of fungicidal growth and metabolism, plant defense reactions and respiration of lesioned tissue, which can be considerably greater consumers of resources than respiration in unaffected tissues. The attack by phytopathogens thus presents a strong impact on several of the plant’s physiological processes, all of which are relevant for production and quality, being that efficiency with fungicide prevents these functional disturbances in the plant. Therefore, the most important contribution provided by the pyraclostrobin molecule to agriculture is derived from its wide range fungicidal activity (Ammermann et al., 2000). However, because the plant absorbs a certain amount of the fungicide applied, changes in the plant’s metabolism and growth may occur, without any relation with the plant’s defense against fungi. Field experiments have revealed that cereals treated with pyraclostrobin show significant increases in production, greater than those due only to its fungicidal effect (Köehle et al., 2003). Thus, the fungicide presents additional effects on the culture’s physiology, which lead to a positive influence in the formation of production. Such effects of fungicides of the strobilurins type were initially described for Kresoxim-methyl and some of the complex dynamic relations in the production’s formation process were widely discussed (Köehle et al., 1997a, 1997b; Clark e Leandro, 1998;
2. Mode of Action in Plants

Pyraclostrobin is a fungicide of strobilurins group and it acts by inhibiting mitochondrial respiration by blocking the transfer of electrons in the III complex (bc1 complex) of the transporting current for mitochondrial electrons (Ammermann et al., 2000). Since the bc1 complex persists in all eucaryotae, at least one partial inhibition in the transportation of electrons must also be expected in plant cells after absorbing the fungicide. As it noted in other molecules of the group and related compounds, the selectivity of respiratory inhibitors of strobilurin type depends less on the difference of sensibility of the mitochondrial complexes from several sources, mainly in terms of bioavailability or abundance in the target site, which is modified by absorption, partition and metabolic breakdown in dynamic processes. Therefore, in the chemical class of strobilurins, the compounds vary in their effects according to their biotechnical properties (Köehle et al., 1994). A transitory influence in the plant mitochondria does not necessarily result in phytotoxicity because the toxicity at the organism level is determined by the importance of mitochondrial respiration for the supply of energy, which varies with environmental conditions and the life stage of the organism (Sauter et al., 1995). For example, strobilurins cause a greater level of cellular damage measured by leakage of the electrolytes, when leaf discs are incubated for some hours in the dark, compared to incubation in the light. Untreated, control leaf discs do not show significant leakage under the same conditions (Köehle et al., 2003).

Although the strobilurins effects in plants has been studied for more than seven years, there is no evidence of any direct interaction of pyraclostrobin with enzymes of receptor systems other than mitochondrial respiration (Köehle et al., 2003).

3. Absorption, Reduction and Assimilation of Nitrogen

The increase in biomass and production, obtained by application of pyraclostrobin, even in plants not affected by fungi, is of special interest for agricultural practices. On the field, in the comparison of other adequate fungicides that control different pathogenic fungi, this may be quantified by remissive spectroscopy (Rouse et al., 1974). The parcels treated with pyraclostrobin show greater values for NDVI = vitality index (Rouse et al., 1974), relating with the increased potential for formation of production.

In contrast with the situation presented by Kresoxim-methyl (Köehle, 1997b), pyraclostrobin only caused a small change in the point of compensation of treated plants’ CO₂. Some results indicate that a transitory increase of the alternative route (AOX) may superimpose the expected reduction in the emission of CO₂ due to the inhibition of mitochondrial respiration. Since an increase in biomass also requires greater assimilation of nitrogen, the NADH-nitrate reductase (NR; EC 1.6.6.1) that catalyzes the first stage of nitrate assimilation, is considered as the relevant target for the effect in production caused by pyraclostrobin (Köehle et al., 2003).

In a previous work, a strong activating effect of NADH-nitrate reductase of Kresoxim-methyl, in the short term, was verified in a simple model system, with floating discs of spinach leaves in buffered solutions containing strobilurin (Glaab and Kaiser, 1999). The reduction of nitrate to nitrite is seen as the limiting step in the assimilation of N and thus highly regulated. Apart from the regulation of transcription and translation, a direct modulation of enzymatic activity is necessary for fast adaptation to the changing environmental conditions (Kaiser, 1999). No direct influence of pyraclostrobin in the activity of in vitro, isolated NADH-nitrate reductase was measured. However, when wheat plants (Triticum aestivum L. cv. Kanzler), produced in hydroponic cultures were pulverized with...
pyraclostrobin, in doses normally used for control of fungi on field, there was an accumulation of nitrate and ammonia in the leaves during the first nocturnal period following application. This was probably due to the fact that the NADH-nitrate reductase was not rendered inactive by darkness as in control plants. Although in vivo levels of nitrate reduction remained unchanged during the day, the in vivo level of reduction increased by about 100% during the nocturnal period. This increase in nitrate reduction persisted for 3 nights after a single application of pyraclostrobin (Köehle et al., 2003).

Köehle et al. (2003) noticed that the absorption in vivo of nitrate was also stimulated by pyraclostrobin, although 7 days after the application, the nitrate content in the tissue of buds was reduced by about 10%, indicating that it had been assimilated for more complex metabolisms. The plants show a clear increase in biomass of about 20%, two weeks after the fungicide’s application.

According to Köehle et al. (2003), nitrate assimilation in plants pulverized with pyraclostrobin was increased, in comparison with control plants, without treated. Neither the relative content of protein nor the C/N ratio were different in control plants treated with the fungicide, indicating that the additional absorption and reduction of nitrate was used to favor the growth of treated plants. This may explain the finding that, frequently, the most prominent effect in the development of wheat is reached when the fungicide is applied during the phase in which nitrogen demand is maximum.

4. Hormonal Changes

The strobilurin Kresoxim-methyl proved to inhibit the biosynthesis of ethylene through reduction of the activity of 1-amino cyclopropane-1-carboxylic acid (ACC)-synthase in tissue of wheat buds (Grossmann e Retzlaff, 1997). This was related to the delay in the senescence of leaves and, as a result, to the prolonged photosynthetic activity of green tissue and a better management of stress (Köehle et al. 1997a; Grossmann e Retzlaff, 1997; Grossmann et al., 1999).

Ethylene may be produced in practically all parts of plant tissue, although the rate of production depends on the type of tissue and the stage of development. Usually, the merismatic regions and the nodal regions are the most active in ethylene production. However, ethylene production increases during leaf abscission of the flower and ripening of fruits. Any type of lesion can induce the biosynthesis of ethylene, including physiological stress caused by inundation, cooling, diseases, temperature or water stress (Taiz e Zeiger, 2004). Ethylene is also recognized as the primary hormonal mediator of plant senescence in stress reactions (Taiz e Zeiger, 1998).

In cultures like wheat, stress by ethylene impairs production, by promoting leaf senescence and the start of premature ripening of the grains, which reduces production of assimilates and the period of grain filling. The key enzyme in biosynthesis of ethylene is ACC-synthase, which converts S-adenosyl-methionine to 1-amino cyclopropane-1-carboxylic acid (ACC) (Abeles et al., 1992).

Köehle et al. (2003) confirm the effect of pyraclostrobin in the activity of ACC-synthase and synthesis of ethylene under conditions of stress and senescence in wheat. Hormonal levels of indol-3-acetic acid (IAA) and abscissic acid (ABA) were also determined. New wheat plants (T. aestivum L. cv. Kanzler) were treated in leaves with pyraclostrobin for 3 hours. This was followed by dry stress, initiated to allow the detached buds to lose fresh weight under conditions of reduced humidity (Grossmann e Retzlaff, 1997). For 1 hour of stress, the fresh weight of detached buds reduced by about 6% while the activity of ACC-synthase increased by about 80 times in relation to the intact, non-stressed buds. According to the authors, pyraclostrobin inhibited the activity of ACC-synthase and the levels of ACC in the tissue in up to 63% at 10^-4 M. On the other hand, pyraclostrobin in a concentration of 10^-9 to 10^-4 M did not change the activity of in vivo ACC-synthase using enzymes extracted from detached buds and subjected to drying. This indicates that the fungicide may inhibit the synthesis of enzymes again.
4.1. Retarded senescence after treatment with pyraclostrobin.

The detached leaves of a plant slowly lose chlorophyll, RNA, lipids and proteins, even if they are kept wet and are supplied with minerals. Such process of programmed aging, which leads to the plant’s death, is called senescence (Taiz e Zeiger, 2004).

After exposing the wheat leaf discs to pyraclostrobin for 48 hours, the loss of chlorophyll, as a parameter of the progression of senescence, was inhibited by a growing concentration of strobilurin (Grossmann e Retzlaff, 1997). Maximum retardation of leaf senescence, with up to 82% of the higher level of total chlorophyll in relation to the control was observed at $10^{-4}$ M of pyraclostrobin. The response to the dose in the retardation of senescence by pyraclostrobin had close correlation with decreasing levels of formation of ACC and ethylene and increase of IAA. Indol-3-acetic acid (IAA) is the commonest form of natural occurrence of phytohormonal auxin (Taiz e Zeiger, 1998).

The endogenous levels of IAA may be the result of pyraclostrobin metabolism because this strobilurin breaks down to the natural precursor of IAA, L-tryptophan in wheat (Köehle et al., 2003). Low levels of auxin are known to retard leaf senescence and to also favor production by stimulating formation of vascular tissue, division of assimilate formation of floral buds and fruit development (Taiz e Zeiger, 1998).

As another effect of pyraclostrobin, it increases concentration of endogenous levels of abscissic acid (ABA) up to a maximum of 269% on the control. ABA inhibits growth and stomatic opening, especially when the plant is under environmental stress, thus improving the utilization of water under conditions of water stress and the adaptation to low temperatures (Taiz e Zeiger, 1998; Grossmann et al., 1999). ABA was originally isolated as a causing factor of abscission, and also promotes leaf senescence, however, in high concentrations (Grossmann, 2000).

4.2. Pyraclostrobin alleviates oxidative stress in plants.

Unfavorable environment (stressing) stimulate the formation of radicals, especially of reactive oxygen forms and increase the oxidative potential in plant tissues (Bartosz, 1997; Wingsle et al., 1999).

In some cereals, mainly susceptible varieties of barley, the reactive oxygen forms may induce the formation of the so-called physiological leaf spot formation (Wu e Tiedemann, 2001, 2002a, 2002b), which causes severe losses in productivity (Baumer et al., 2001).

Resistant plants respond to oxidative stress with increase in the activity of antioxidative enzymes, such as superoxide-dismutases, catalases and peroxidases (Larson, 1997).

In field tests conducted in Worms, Palatinate, Germany, winter barley plants treated with pyraclostrobin did not develop symptoms, while flag leaves and lower leaves of untreated plants were covered by symptoms of physiological leaf spots, indicating some blocking reactions of radicals that opposed oxidative stress. When the activity of peroxidase in the flag leaf was evaluated, the plants treated with pyraclostrobin showed almost doubled enzymatic activity, which can contribute toward tolerance to stress. It is interesting that this effect, established five days after treatment with fungicide, persisted for over four weeks (Köehle et al., 2003).

Based on the findings, Köehle et al. (2003) concluded that pyraclostrobin (F 500) altered the status of phytohormones in wheat bud tissues. The most remarkable change was the inhibition of ethylene biosynthesis by the reduction of the activity of ACC synthase. Together with the increase in endogenous auxin, this change in hormonal balance would explain the retarded senescence of leaves and enhancement in the tolerance to stress. Also, pyraclostrobin stimulated the levels of ABA, and the authors believe that this might favor tolerance to cold and adaptation to conditions of water shortage. Increase in the antioxidative capacity,
following treatment with pyraclostrobin, might be involved in the prevention of symptoms of physiological leaf spot.

5. Induction of Resistance to Virus

Several studies in plants refer to the involvement of mitochondria in the defense response induced by pathogens. The latest studies showed that salicylic acid (AS) promotes induced resistance to TMV, being sensitive to salicylhydroxamic acid (SHAM), an alternative inhibitor of the oxidase pathway in mitochondria. In addition, the inhibitors of respiration antimycin A and cyanide induced accumulation of translated oxidase and resistance to TMV (Xie e Chen, 2000).

A model of induction of resistance to viruses was suggested by Singh et al. (2003) (Figure 1). According to the authors, it is known that AS can stimulate resistance to viruses using at least two pathways of transduction. It can stimulate increase in the transcription of RdRp1, which is found increasing the covering of viral RNA through mechanisms based on RNAi (Xie et al., 2001; Yu et al.; 2003). Alternatively, AS can inhibit respiration in the chain of transportation of electrons (Xie e Chen, 1999), leading to an increase in the reactive oxygen species (ROS) in mitochondria.

Other inhibitors of respiration in the transportation of electrons such as antimycin A (AA), cyanide (Chivasa e Carr, 1998) or strobilurins type fungicides (Herns et al., 2002) can also cause an increase in ROS. The amplitude and duration of this increase in mitochondrial ROS will be under negative regulation through AOX (alternative oxydase). This might explain why in a transgenic plant with increase or decrease of AOX, the capacity to signalize is respectively reduced or amplified (Gilliland et al., 2003). Signals of mitochondrial ROS can be detected by a protein sensor that is still unidentified, by means of a mechanism of thiol/disulfite change, as proposed by Dutilleul et al. (2003). Subsequent signals are proposed to the induction of nuclear genes that affect movement and viral replication. Although not shown in this diagram, the expression of the Aox in the nucleus is transitory and stimulated by inhibition of the chain of electron transportation, leading to increase in the accumulation of AOX in the mitochondria.

**Figure 1** - A model of induction of resistance to viruses by salicylic acid (AS). Singh et al. (2003)

These findings and the effects described above indicate that the pyraclostrobin fungicide can also increase the ability of certain plants to resist phytopathogenic attack. Due to the fungicidal activity of pyraclostrobin, such increase in resistance of plants could hardly be proved for a resistance to phytopathogenic fungi, therefore the effect pyraclostrobin on resistance was investigated by one of the best models of the system for studying interactions, specifically interaction between a tobacco mosaic virus (TMV) and tobacco plants containing the N gene. Due to the presence of the N gene of resistance to diseases in tobacco plant cultivations, the spread of TMV is restricted to a small zone around the site of infection (Matthews, 1991).

The restriction of TMV is followed by the localized death of the host tissue’s cells and results in the formation of visible necrotic lesions (hypersensitivity response). After the formation of lesions, the tobacco plants develop a type of acquired resistance, added to the subsequent TMV attack, close to the area of primary infection and often throughout the plant (Dempsey et al., 1999; Ryals et al., 1996).
The resistance added to the tobacco against TMV infection becomes obvious by the formation of necrotic lesions that are substantially smaller than those formed in tobacco plant leaves that were not pre-infected by TMV (Köehle et al., 2003).

To ascertain if the pre-treatment of tobacco plants (cv. Xanthi nc) with pyraclostrobin influences its resistance to TMV attack, a prolonged infiltration of fungicide in tobacco leaves was found to increase its resistance to TMV infections. The induced expression of the TMV PR-1 genes and accumulation of PR-1 proteins were clearly induced much earlier in plant leaves treated with pyraclostrobin than in leaves infected by TMV of plants that were not previously treated with pyraclostrobin. Therefore, the fungicide from the strobilurins group, pyraclostrobin, in addition to serving as a potent fungicide, probably also protects the plants by increasing their inherited capacity to activate a cellular defense response, merely induced by a later attack of pathogens, as was shown by the activation of PR-1 genes in tobacco plants infected by TMV (Herms et al., 2002; Köehle et al., 2003).

According to Köehle et al. (2003), the added resistance becomes obvious by a substantial reduction in the size of TMV lesions, not due to the inhibiting effect of pyraclostrobin on the TMV’s potential to infect tobacco. Interestingly, in contrast to the induction of added resistance to TMV by the salicylic acid compound, the resistance induced by pyraclostrobin was not associated with an accumulation of the so-called proteins related to pathogenesis (PR-1) (Herms et al., 2002; Köehle et al., 2003) which, although its enzymatic activity and physiological function are still obscure, serve as reliable molecular markers of added resistance to diseases in various plants, including tobacco.

Pyraclostrobin presents prominent physiological effects on plants, and other, still unknown effects may also occur (Köehle et al., 2003). Provided it is unlikely that the molecule interacts specifically with many different biochemical targets, we have to imagine how the reactions may be connected or if there is an initial and central effect that could set off a cascade of consequences. A possible explanation for such phytohormonal changes in wheat plants induced by pyraclostrobin has already been mentioned, that the metabolism of the compound results in an increase in the precursor of AIA, L-tryptophan. This however cannot explain the effects of nitrogen assimilation and the induction of resistance against viral attack.

A new perspective was opened by some preliminary results showing that treatment with fungicide induces the formation of nitric oxide (NO). For this reason, the authors now propose other sequential hypothesis to explain the multiple physiological effects of pyraclostrobin, still assuming that the fungicide’s primary mode of action in plants is partial and transitory inhibition of the respiratory chain in mitochondria (Figures 2a and 2b).

Inhibition of mitochondrial respiration by pyraclostrobin activates the alternative oxydase pathway (AOX), decreases cellular levels of ATP while [H⁺] in the cytosol increases, both resulting in an activation of NADH-nitrate reductase (NR) (Glaab e Kaiser, 1999).

Activation of NR results, transitorily, in increase in the nitrite levels and may enhance plant growth when N assimilation is a level limiter. There is also an increase in the production of N via NR. In addition, for the nitric oxide synthase (NOS) process, there is an alternative pathway of NO production in plants, NR product with NADH-nitrite as substrates (Yamasaki, 1999).

Provided that nitric oxide competes with...
oxygen for cytochrome oxydase, the oxygen consumption through the cytochrome pathway in mitochondrial respiration is inhibited (Millar e Day, 1996). For this reason, the initial effect of pyraclostrobin in plants could really be self-stimulating. Low concentration of ATP also sensitizes the mitochondrion by induced formation of pores for Ca^{2+} (Jones, 2000), with significance in the recognition of a pathogenic attack (Jabs e Shusarenko, 2000). This could contribute for an “initial” effect, accelerating the defensive reactions against TMV infections, as described above.

![Diagram](image)

**Figure 2b)** - Influence of signaling in plant cells via nitric oxide (NO).

It is suggested that nitric oxide and/or peroxynitrites can inhibit the activities of ACC synthase and ACC oxydase, key enzymes of ethylene biosynthesis, by oxidative inactivation of its co-factors. Thus, nitric oxide can remarkably reduce the rate of C2H4 emission, with all the implications, including management of stress, “greening effect” and inhibition of premature senescence. Nitric oxide is more commonly considered to be a central component of the general adaptation syndrome (GAS) mechanism in plant tolerance to stress (Leshem, 2000).

Also, NO is known to interact with G-proteins, leading to the generation of cGMP and ADAP cyclic ribose as second messengers in plant defensive response (Bolwell, 1999; Broillet, 1999; Wendehenne *et al.*, 2001). Nitric oxide also seems to be involved in the decreasing signaling closely linked to SA. As an example, treatment of tobacco leaves with nitric oxide induced a significant increase in the SA endogen required for induction of the PR-1 gene (Durner, 1998). The relationship between nitric oxide, SA and reactive oxygen species (ROS) in the activation of defense genes and/or induction of death of host cells are better described as a self-simplifying process during which redox signaling through nitric oxide and ROS is stimulated by SA (Van Camp, 1998). SA inhibits the formation of jasmonate, which, by its turn, also results in decrease in the formation of ethylene and less peroxidation of lipids. All this can also contribute to the management of stress, which was certainly observed in the treatment with pyraclostrobin of non-infected plants.

### 6. Final Considerations

Considered as a whole, nitric oxide acting in the right place, at the right time and in the right quantities, would not be only considered as a critical element in the development of oxidative flow and resistance against pathogens and stress, but it could also acts as key element and messenger in physiological effects promoted by the fungicide pyraclostrobin, connecting the different levels of its appearance. There are increasing evidences that plant mitochondria perform a central role as stress sensors. Additional research will be needed to understand how the modulators of mitochondrial activity, including this new group of fungicides, can contribute toward the defensive reactions in plants under biotic and abiotic stresses. From a practical standpoint, Bergmann *et al.* (1999) affirm that, in addition to protection against phytopathogenic fungi, such activity of resistance, inherent to plants with stress, could increase not only productivity but also the quality of products.
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