7

Physiological Effects of the Strobilurin Fungicide F 500 on Plants

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During the last decade of intensive research on the fungicidal properties of strobilurins evidence for direct influences on the physiological processes of plants not infected or challenged by pathogens has also increased. This activity is referred to as the 'Physiological Effect'.

The 'Physiological Effects' of the new BASF strobilurin F 500 (common name: pyraclostrobin) have been studied at different levels of complexity, ranging from the frequently mentioned greening effect and amelioration of stress factors observed in the field and under controlled conditions, down to influences on hormonal regulation and carbon and nitrogen assimilation by the plant. A working hypothesis which unites these multiform observations under a primary molecular mode of action will be discussed.

Introduction

From a plant physiological perspective, agronomic practice aims to maximize the photosynthetic efficiency of crops and to channel its products into yield formation, rather than into other, non-productive energy sinks. Fungal infections impair crop efficiency by greatly reducing the area of active photosynthetic tissue and by inhibiting the translocation of assimilates from the sources of their production to areas of plant growth and yield deposition. Infection also diverts assimilates into the non-productive sinks of fungal growth and metabolism, plant defense reactions and wound respiration, which can be a considerably greater consumer of resources than respiration in non-infected tissues. Fungal pathogen attack thus has a deep impact on different physiological processes of the plant, all of which are relevant to crop yield and quality, and each successful fungicide treatment prevents such disturbances of plant functions. Consequently, the most important contribution the new strobilurin fungicide F 500

Modern Fungicides and Antifungal Compounds III

makes towards achieving the aims of the agronomist derives from its outstanding, broad range fungicidal activity (Ammermann et al., 2000).

However, due to the fact that a certain amount of the applied fungicide is taken up by the plant, changes in metabolism and growth may occur, which are not related to plant defence against fungi. Field trials have revealed that F 500–treated cereals show yield increases significantly greater than those accounted for by its fungicidal effects only. Thus, the fungicide has additional effects on crop physiology which lead to a positive influence on yield formation. Such effects of a strobilurin type fungicide were first described for kresoxim-methyl and some of the complex, dynamic relationships within the processes of yield formation have been discussed previously (Koehle et al., 1997a, 1997b; Clark and Leandro, 1998; Grossmann and Retzlaff, 1997; Retzlaff, 1995).

The goal of this paper is a re-examination of the most prominent physiological effects for the new strobilurin F 500 and to propose a model linking the various processes altered by F 500 treatment.

Results and Discussion

The primary mode of action of F 500 in plant cell metabolism

F 500 is a strobilurin type fungicide and so its mode of action is the inhibition of mitochondrial respiration by blocking electron transfer in complex III (bc1 complex) of the mitochondrial electron transport chain (Ammermann et al., 2000). Since the *bc*1 complex is conserved in all eukaryotes, at least a partial inhibition of mitochondrial electron transport should be expected also in plant cells following uptake of F 500. As shown previously for kresoxim-methyl and related compounds, the selectivity of strobilurin type inhibitors of respiration depends less on differential sensitivity of mitochondrial complexes from different sources, but strongly on terms of bioavailability or abundance at the target site, which is modified by uptake, partitioning and metabolic degradation as dynamic processes. So within the class of strobilurin chemistry, compounds vary in their effects on plants according to their biokinetic properties (Koehle et al., 1994). A transient influence on plant mitochondria does not necessarily result in phytotoxicity because the toxicity at the level of organism is determined by the importance of mitochondrial respiration for energy supply which varies with environmental conditions and the life stage of the organism (Sauter et al. 1995). For example, strobilurins cause a much higher rate of cell damage measured by leakage of electrolytes when leaf disks are incubated for some hours in the dark compared to illuminated incubation. Untreated control leaf disks did not show significant leakage under the same conditions (Koehle, unpublished results).

Although we have studied the effects of strobilurins on plants for more than seven years, there is no evidence for any direct interactions of F 500 with enzymes or receptor systems other than mitochondrial respiration.

Growth stimulation by F 500 as related to nitrate uptake, reduction and assimilation in wheat plants

Of special interest to agricultural practice is the increase in biomass and yield achieved by application of F 500, even in plants not infected by fungi. In the field, in comparison to other suitable fungicides which control the abundant fungal pathogens, this can be quantified by remission spectroscopy (Rouse et al., 1994). Plots treated with *F 500* showed higher values for NDVI (for method see Rouse et al., 1974), (Figure1a, 1b), correlating with increased potential for yield formation (Figure 2).

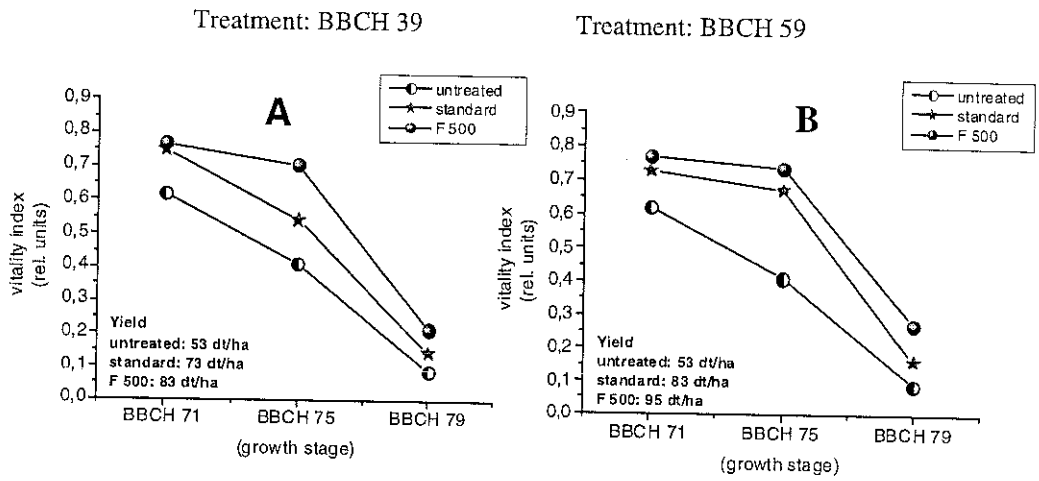


Figure 1. Increase of vitality index and yield of wheat plants by treatment with F 500 at different growth stages

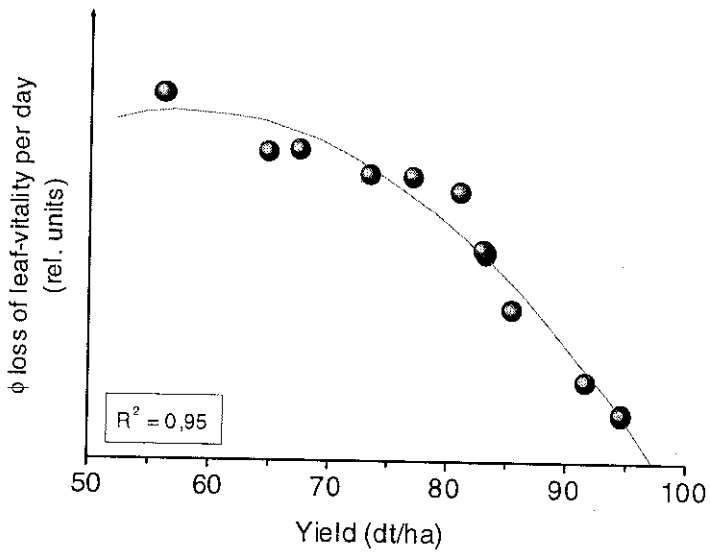
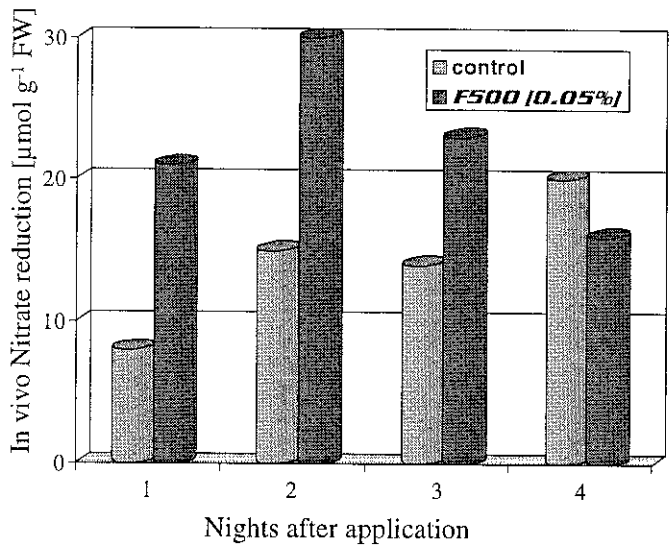


Figure 2. Relationship between average (Φ) loss of leaf–vitality per day and yield (time course: growth stage BBCH 71 – 79)

In contrast to the situation with kresoxim–methyl (Koehle, 1997b), F 500 caused only little change in the CO_2-compensation point of treated plants (Retzlaff, unpublished). Some results indicated that a transient increase of the alternative pathway (AOX) may overlay the expected reduction in CO_2 emission due to the inhibition of mitochondrial respiration (Koehle, unpublished). Since an increase in biomass requires also higher nitrogen assimilation, we considered NADH-nitrate reductase (NR; EC 1.6.6.1) which catalyzes the first step in nitrate-assimilation as the relevant target for the yield effect of *F 500*. In previous work, a strong short-term NR-activating effect of kresoxim-methyl was verified in a simple model system with spinach leaf discs floating on buffer solution containing the strobilurin (Glaab and Kaiser, 1999). The reduction of nitrate to nitrite is regarded as the rate-limiting step in N-assimilation and therefore highly regulated. Besides transcriptional and translational regulation, direct modulation of enzyme activity is required for rapid adaptation to changing environmental conditions (for review see Kaiser, 1999). No direct influence of *F 500* on the activity of isolated NR *in vitro* was measured. However, when hydroponically grown wheat plants (*Triticum aestivum* L. cv. Kanzler) were treated by spray application at rates of *F 500* normally used for fungal control at the field site, nitrite and ammonia accumulated in the leaves during the first night period following application. This was probably due to the fact that NR was not dark-inactivated as in control plants. Although *in vivo* nitrate reduction rates were unaltered during day time, the *in vivo* reduction rate was increased about 100 % during the night periods. This enhancement in nitrate reduction persisted for 3 nights after a single application of F 500 (Figure 3).



Short Term Effect:

- A few hours after application of **F 500** onto wheat leaves the activity of NR in the dark increased for more than 70%.
- Under these conditions NR-activity seems no longer to be the rate limiting step of Nitrogen-Assimilation.

Figure 3. Increase of nitrate-reductase in vivo

Nitrate uptake *in vivo* was also stimulated by F 500, although, 7 days after application the nitrate content in the shoot tissue was decreased about 10 %, indicating that it had been assimilated into more complex metabolites. (Figure 4 a). Plants

showed a clear increase in biomass of about 20 %, 2 weeks after the application of the fungicide (Figure 4 b).

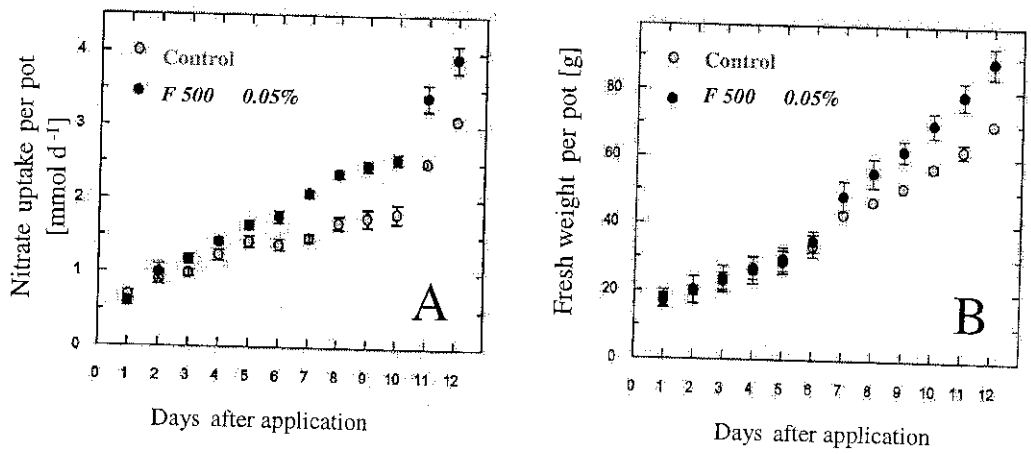


Figure 4. Stimulation of nitrate uptake in vivo and of plant growth. Wheat plants were grown in hydroponic culture.

A. After a delay, the root uptake of nitrate increased when leaves were treated with F 500.

B. B. The increase of nitrate uptake was followed by enhanced plant growth.

Summarizing the observations described until now, the nitrate assimilation in plants sprayed with F 500 was enhanced compared to controls. Neither the relative content of protein nor C/N-ratios were different in control and F 500–treated plants, indicating that the additional uptake and reduction of nitrate was used for enhanced growth of sprayed plants. This could explain the observation that frequently the most prominent effect on wheat development is achieved when *F 500* is applied during the phase when the plant's demand for nitrogen is greatest.

Phytohormonal changes in wheat plants induced by F 500 and coping of stress

The strobilurin kresoxim-methyl was found to inhibit ethylene biosynthesis via a reduction in endogenous 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity in wheat shoot tissue (Grossmann and Retzlaff, 1997). This has been linked with delayed leaf senescence and consequently prolonged photosynthetic activity of the green tissue and improved stress management (Grossmann and Retzlaff, 1997; Koehle et al., 1997a; Grossmann et al., 1999). Ethylene is known to be the primary hormonal mediator of plant senescence and stress reactions (Taiz and Zeiger, 1998). In crops like wheat, stress ethylene impairs yield through a promotion of leaf senescence and the initiation of premature ripening of the grain which diminish the production of assimilates and the duration of grain filling. The key enzyme in ethylene biosynthesis is ACC synthase which converts S-adenosyl-methionine to ACC (Abeles et al., 1992). Therefore, the effects of F 500 on ACC synthase activity and ethylene synthesis were characterized under stress and senescence conditions in wheat. In addition, phytohormone levels of indole-3-acetic acid (IAA) and abscisic acid (ABA) were

determined. Young wheat plants (Triticum aestivum L. cv. Kanzler) were foliar-treated with F 500 for 3 h. Subsequently, drought stress was initiated by allowing detached shoots to lose fresh weight under reduced humidity conditions (Grossmann and Retzlaff, 1997). During 1 h of stress, the fresh weight of detached shoots decreased by ca. 6 %, while ACC synthase activity increased about 80-fold relative to intact, non-stressed shoots. *F 500* effectively inhibited ACC synthase activity and ACC levels in the tissue by up to 63 % at 10^{-4} M. In contrast, *F 500* in concentrations of 10^{-8} to 10^{-4} M did not alter ACC synthase activity *in vitro* using enzyme extracted from detached shoots subjected to drought (results not shown). This indicates that *F 500* may inhibit *de novo* enzyme synthesis (Figure 5).

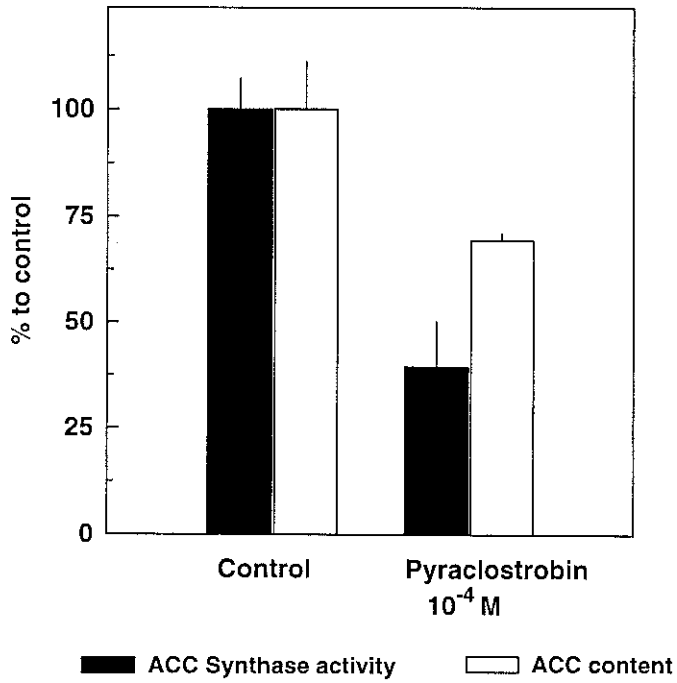


Figure 5. Inhibition of ACC synthase activity.
Effects of *F 500* on 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity and ACC levels in shoots from wheat. Plants were foliar-treated with the compound for 3 h. Shoots were isolated and water-stressed. Vertical bars represent SE of the mean.

Delayed senescence following F 500 treatment

After exposure of wheat leaf discs to *F 500* for 48 h (Grossmann and Retzlaff, 1997), chlorophyll loss as a parameter for senescence progression was inhibited with increasing strobilurin concentration (Figure 6). Maximum retardation of leaf senescence, with up to 82 % higher level of total chlorophyll relative to control, was observed at 10-4 M *F 500*. The dose-response of delayed senescence by *F 500* closely correlated with decreased levels of ACC and ethylene formation and a rise in IAA (Figure 7). IAA is the most common naturally occurring form of phytohormonal auxin (Taiz and Zeiger, 1998). The increase in endogenous levels of IAA could be a result of F 500 metabolism, because this strobilurin degrades to the natural IAA-precursor L-

tryptophan in wheat (K. Reinhard and R. Doetzer, BASF Limburgerhof; personal communication). Low levels of auxin are known to delay leaf senescence and additionally favour yield through stimulation of vascular tissue formation, assimilate partitioning, floral bud formation and fruit development (Taiz and Zeiger, 1998). As a further effect of *F 500*, concentration-dependent increases to a maximum of 269 % of the control were observed in endogenous ABA levels (Figure 6). ABA improves the plant water status under drought conditions and the adaptation to cold temperatures (Taiz and Zeiger, 1998; Grossmann et al., 1999). ABA also promotes leaf senescence, albeit, only at high concentrations (Grossmann, 2000).

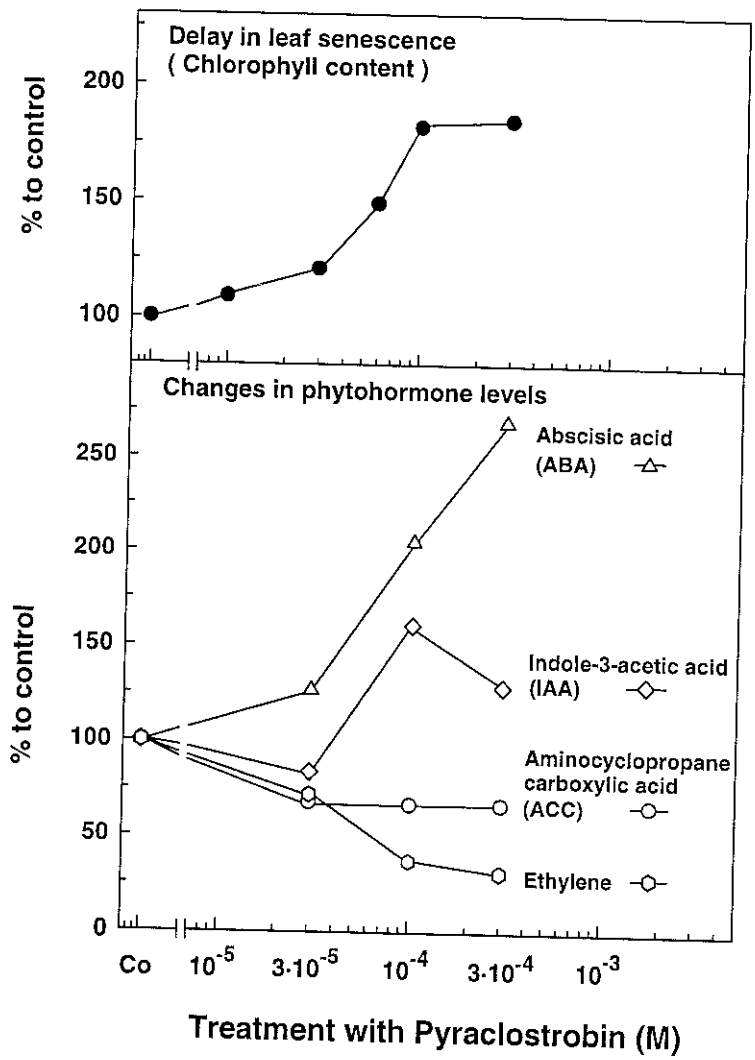


Figure 6. Delay in leaf senescence and changes in phytohormone levels.
Effects of F 500 on total chlorophyll content, ethylene formation and immunoreactive phytohormone levels in senescing leaf discs of wheat after 48 h of treatment in the dark.

F 500 alleviates oxidative plant stress

Unfavourable environments ('stressors') enhance the formation of radicals, especially of reactive oxygen species, and increase the oxidative potential in plant tissues (Bartosz, 1997; Wingsle et al., 1999). In some cereals, particularly in susceptible varieties of barley, this can induce the formation of so called physiological leaf spot disease (Wu and Tiedemann, in press) which causes severe losses in yield (Baumer et al., 2001). Resistant plants respond to oxidative stress with e.g. increased activities of anti-oxidative enzyme, such as superoxide dismutases, catalases and peroxidases (Larson, 1997). In a field trial (Worms, Palatinate, Germany), winter barley plants treated with F 500 did not develop such symptoms while flag and lower leaves from untreated plants were covered with physiological leaf spot symptoms (Figure7a), indicating that some radical-eliminating reactions counteracted this oxidative stress. When the peroxidase activity in flag leaf tissue was measured, plants treated with F500 showed nearly twofold enzyme activity (Figure 7 b) which may contribute to stress coping. Interestingly, this effect was already established five days after fungicide treatment and persisted for more than 4 weeks.

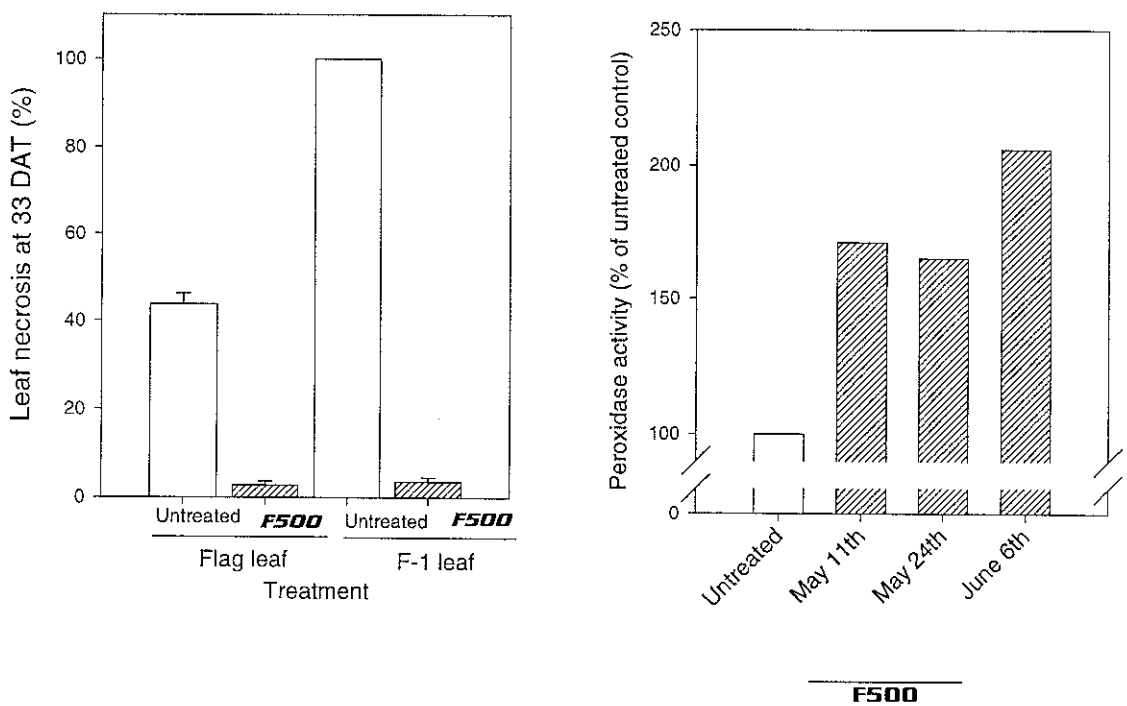


Figure 7. Influence of F 500 on leaf necrosis and peroxidase activity

A. Physiological leaf spot symptoms scored as leaf necrosis 33 days after fungicide treatment. F 500 was applied at May 6th, (growth stage EC49) as a suitable formulation for use in cereals.

B. Guaiacol peroxidase activity was determined from flag leaf extracts 5 days and 30 days after fungicide treatment. Physiological leaf spot symptoms developed not before 24 days after treatment (May 30th).

In conclusion, F 500 changes the phytohormone status in wheat shoot tissue. The most remarkable alteration was the inhibition of ethylene biosynthesis through a reduction in ACC synthase activity. Together with an increase in endogenous auxin,

this shift in the hormonal balance may explain delayed leaf senescence and improved stress tolerance. In addition, F 500 stimulated ABA levels. This could favour cold tolerance and adaptation to water deficit conditions. The increase in anti-oxidative capacity following treatment with F 500 could be involved in preventing physiological leaf spot symptoms.

F 500 enhances the resistance of tobacco against Tobacco Mosaic Virus

In plants, the involvement of mitochondria in pathogen-induced plant defence responses has long been implicated in various studies. More recently it has been demonstrated that the SA-induced tobacco resistance to TMV is sensitive to SHAM, an inhibitor of the alternative oxidase pathway in mitochondria. Moreover, the respiratory inhibitors antimycin A and cyanide induced alternative oxidase transcript accumulation and resistance to TMV (for review see Vanlerberghe and McIntosh, 1994, 1996).

These observations and the effects shown above, indicated that *F 500* may also enhance the ability of certain plants to resist pathogen attack. Due to the extraordinary fungicidal activity of F 500, such an enhancement of plant resistance against fungal infections by indirect or induced mechanisms could hardly be proved. So the effect of F 500 on plant disease resistance in one of the best characterized model systems to study plant-pathogen interactions, namely the interaction between tobacco mosaic virus (TMV) and N gene-containing tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) was investigated. Due to the presence of the disease resistance N gene in plants of this tobacco cultivar, TMV spread is restricted to a small zone around the infection site (Matthews, 1992). TMV restriction is accompanied by localized cell death of host tissue and results in the formation of visible necrotic lesions (hypersensitive response). After lesion formation, tobacco plants develop an enhanced (acquired) resistance to further TMV attack near the area of primary infection and often throughout the plant (Dempsey *et al.,* 1999; Ryals *et al.,*1996). The enhanced resistance of tobacco against TMV infection becomes obvious in the formation of necrotic lesions that are substantially smaller than those formed on leaves of tobacco plants which have not been pre-infected by TMV.

By investigating whether pre-treatment of tobacco (cv. Xanthi nc) plants with F 500 influences their resistance to TMV attack, it was found that a prolonged infiltration of F 500 into tobacco leaves enhanced their resistance to TMV infection (Herms *et al.,* unpublished). The enhanced TMV resistance became obvious in a substantial reduction in TMV lesion size and was not due to an inhibitory effect of F 500 on the potency of TMV to infect tobacco. Interestingly, in contrast to the induction of enhanced TMV resistance by the natural signalling compound salicylic acid, the F 500-induced TMV resistance was not associated with an accumulation of so-called pathogenesis-related (PR)-1 proteins (Herms *et al.,* unpublished). Although their enzymatic and physiological function is still unclear PR proteins serve as reliable molecular markers for enhanced disease resistance in various plants, including tobacco.

Impressively, the TMV-induced expression of the *PR-1* genes and the associated accumulation of PR-1 proteins were induced much earlier in leaves of F 500 pre-treated plants than they were in TMV-infected leaves of untreated plants (Figure 8; Herms *et al.,* unpublished). Thus, the strobilurin fungicide F 500, in addition to serving

as a highly potent fungicide, probably also protects plants by augmenting their inherited capacity to activate cellular defense responses that are only induced upon further pathogen attack as has been shown for the activation of PR-1 genes in TMV-infected tobacco plants.

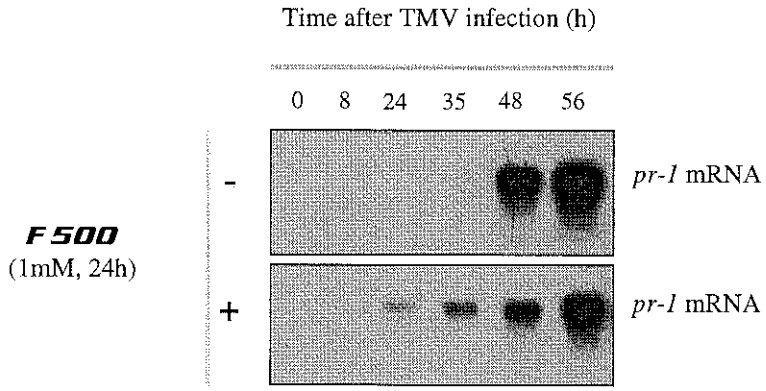


Figure 8. Pretreatment with F 500 causes an acceleration of TMV-induced PR-1 gene expression in tobacco

One half of a leaf of a 6-week-old tobacco (cv. Xanthi nc) plant was infiltrated with water (-) while the second half of the leaf was infiltrated with an aequous solution of *F 500* (+). 24 h later, the leaf has been infected, on both halves, with a solution of TMV (1,5 µg/ml virus protein in phosphate buffer). The accumulation of *PR-1* mRNA was assayed at the indicated time points post TMV infection by Northern blotting analysis with a tobacco *PR-1*-specific cDNA probe.

Conclusion

A draft model to explain the manifold physiological effects of F 500 could be as follows: As the above-mentioned examples demonstrate, F 500 has significant physiological effects on plants and there may be even more not yet known. Since it is unlikely that the molecule interacts non-specifically with many different biochemical targets or receptors, we have to wonder how the reactions could be linked or whether there is an initial and central effect which could initiate a cascade of consequences. A possible explanation for the phytohormonal changes in wheat plants induced by F 500 has already been mentioned above, in that metabolism of the compound results in an increase of the IAA-precursor L-tryptophan. However, this could not explain the effects on N-assimilation and the induction of resistance against viral attack. A new perspective was opened by some preliminary results showing that treatment with F 500 induces the formation of NO. Due to this, we now propose another working hypothesis to explain the manifold physiological effects of F 500, still assuming that the primary mode of physiological action of F 500 in plants is the partial and transient inhibition of the respiratory chain in mitochondria.

Figure 9a summarizes the postulated **primary biochemical reactions** in plant cells:

- Inhibition of mitochondrial respiration by F 500 activates the AOX pathway, it decreases cellular levels of ATP while $[H^+]$ in the cytosol increases, both resulting in an activation of NR (for proposed mode of action see Glaab and Kaiser, 1999).
- Activation of NR results transiently in increased nitrite levels and can improve plant growth when the N-assimilation is rate limiting. Also there is an increased NO-production via NR. In addition to the NOS-mediated process, this is an alternative pathway of NO production in plants and a by product of NR with NADH and nitrite as substrates (Yamasaki, 1999).
- Since NO competes with oxygen for cytochrome oxidase, oxygen consumption via the cytochrome pathway in mitochondrial respiration is inhibited (Millar and Day, 1996). Therefore, the initial effect of *F 500* in plants could be even self-enhancing. Low cellular concentrations of ATP also sensitises the mitochondria for Ca^{2+} induced pore formation (Jones, 2000), playing a role in recognition of pathogen attack (for review see Jabs and Slusarenko, 2000). This could contribute to the "priming" effect, speeding up the defence reactions against TMV infection as described above.

Figure 9b describes the postulated influence on **signalling in plant cells mediated by NO**:

- It is suggested that NO and/or peroxynitrites may inhibit the activities of ACC synthase and ACC oxidase, key enzymes of ethylene biosynthesis, by oxidative inactivation of their cofactors. Thus, NO may markedly reduce the rate of C_2H_4 emission, with all the implications thereof, including stress management, "greening effect" and inhibition of premature senescence. More generally NO is considered to be a central component of the GAS mechanism in plant stress tolerance (for review see Leshem, 2000).
- In addition, NO is known to interact with G-proteins, leading to the generation of cGMP and cyclic ADP ribose as second messengers in plant defence responses (for review see Bolwell, 1999; Broillet, M-C., 1999; Wendehenne et al., 2001). NO also seems to be involved in downstream signalling, closely linked to SA. As an example, treatment of tobacco leaves with NO induced a significant increase in the endogenous SA required for PR-1 gene induction (Durner, 1998). The relationships between NO, SA and ROS in the activation of defense genes and/or induction of host cell death are best described as a self-amplifying process during which redox signalling through NO and ROS is enhanced by SA (Van Camp, 1998). SA inhibits jasmonate formation, which, in turn, also results in decreased ethylene formation and less lipid peroxidation. All this can also contribute to stress management, as, indeed, is observed upon treatment of non-infected plants with F 500.

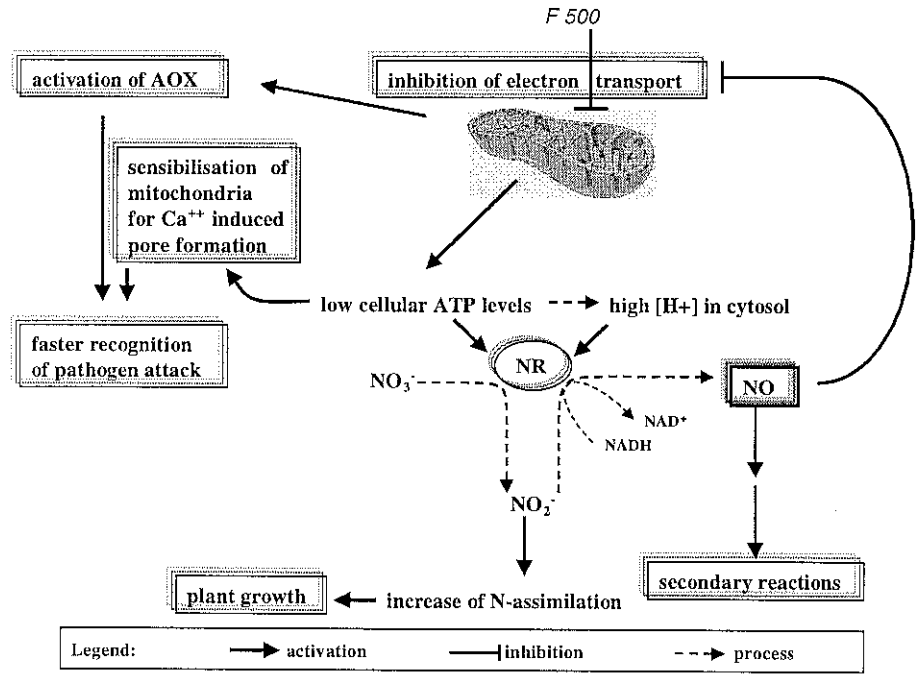


Figure 9 a. Primary biochemical reactions

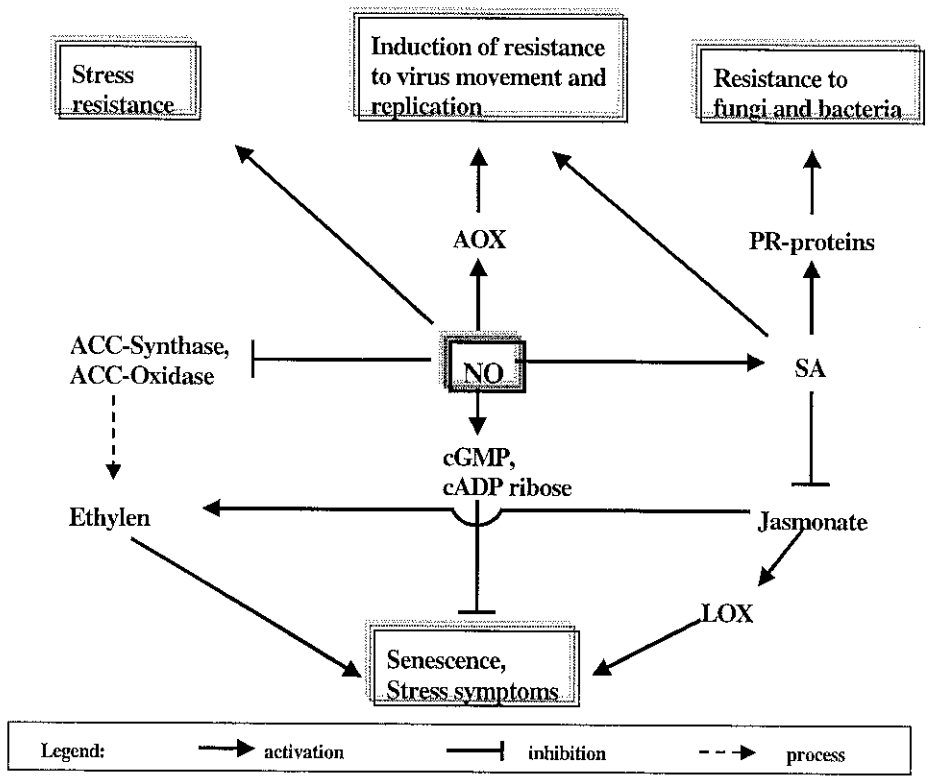


Figure 9 b. Influence on signalling in plant cells via NO

Taken together, NO in the right place, at the right time and in the right amounts is not only reported to be a critical element in the development of the oxidative burst and resistance against pathogens and stress, but could also act as the central switch and messenger in the physiological effects of F 500, linking the different levels of their appearance. There is increasing evidence that plant mitochondria play a central role as stress sensors. Further research is needed to understand how modulators of mitochondrial activity, including the new fungicide F 500, can contribute to the plants sensing of both biotic and abiotic stress and its defence reaction against. From a practical viewpoint, in addition to the excellent direct protection against fungal pathogens, such an activation of the plant's own stress resistance could improve not only yield but also the quality of products (for review see Bergmann et al., 1999).

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Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid; AOX = alternative oxidase pathway of mitochondrial respiration; *F 500* = trade name for Pyraclostrobin; GAS = General Adaption Syndrome; HR = hypersensitive reaction; NDVI = vitality index [NDVI = (NIR-R)/ NIR + R); NIR = 660 nm - 680 nm; R = 840 nm - 860 nm]; NO = nitric oxide; NOS = nitric oxide synthase, E.C.1.14.13.39; NR = NADH-nitrate reductase. E.C. 1.6.6.1; SA = salicylic acid; ROS = reactive oxygen species; SHAM = salicylhydroxamic acid; TMV = Tobacco mosaic virus;

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