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Short Communication

Regulation of Phytohormone Levels, Leaf Senescence and Transpiration by the Strobilurin Kresoxim-methyl in Wheat (*Triticum aestivum*)[1]

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Summary

Using leaf discs and intact wheat plants (*Triticum aestivum* L.), the physiological effects of the strobilurin-type fungicide kresoxim-methyl were studied in relation to induced phytohormonal changes. Dose-response experiments revealed that kresoxim-methyl shifted the hormonal balance, favouring cytokinins particularly of the dihydrozeatin riboside-type, as opposed to ethylene and its biosynthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC). This was closely correlated with delayed leaf senescence. Kresoxim-methyl was shown to inhibit the induction of ACC synthase in ethylene formation. In addition, kresoxim-methyl caused an up to two-fold increase in endogenous levels of abscisic acid, relative to the control. Concomitantly, the stomatal aperture and water consumption of plants were reduced. It is suggested that kresoxim-methyl changes the hormonal constellation in wheat which leads to delayed leaf senescence and water-conserving effects.

Key words: Abscisic acid, cytokinins, ethylene biosynthesis, fungicidal strobilurin, kresoxim-methyl, leaf senescence, stomatal behaviour, transpiration, Triticum aestivum, water consumption.

Abbreviations: ABA = (+) abscisic acid; ACC = 1-aminocyclopropane-1-carboxylic acid; DZR = dihydrozeatin riboside; IAA = indole-3-acetic acid; ZR = trans-zeatin riboside.

Introduction

Kresoxim-methyl is a new fungicide derived from the fungal secondary metabolite strobilurin A which inhibits mitochondrial respiration by blocking electron transfer at the cytochrome-bc_1 complex (Sauter et al., 1995; Anke, 1997). In addition to its direct effect on the fungus, kresoxim-methyl was found to induce non-fungicidal physiological changes in wheat (Grossmann and Retzlaff, 1997). These include a darker green appearance of the leaves, delayed leaf senescence, increased stress tolerance and favoured plant biomass and corn production (Grossmann and Retzlaff, 1997). A model of the physiological events is proposed which includes a dual role of kresoxim-methyl (Grossmann and Retzlaff, 1997). Kresoxim-methyl improves the net photosynthetic efficiency of the plant through a transient inhibition of mitochondrial plant respiration. Furthermore, kresoxim-methyl influences the hormonal status of the plant through a bioregulatory auxin-like activity. This was characterized by the physiological action profile in several bioassays, including shoot formation in tobacco stem explants, anticytokinin activity in *Lemna* (Grossmann and Retzlaff) and auxin-like dose dependency of root formation in germinating wheat seeds (unpublished result). Like auxins, applied at low concentrations, 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). This has been linked with delayed leaf senescence and improved stress management (Grossmann and Retzlaff, 1997). However, the response was not identical to that of auxins since high auxin concentrations normally

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stimulate ethylene formation, whereas kresoxim-methyl inhibited at all concentrations tested and did not induce epinastic deformations (Grossmann and Retzlaff, 1997). Ethylene is known as the primary mediator of plant senescence and stress reactions (Abeles et al., 1992; Nam, 1997). However, extended longevity of leaf tissue has been shown to be determined by a shift in the hormonal balance of a promoter (i.e. ethylene) and an inhibitor (i.e. cytokinin) of senescence (Nooden and Leopold, 1988; Grossmann et al., 1991). Besides its senescence-delaying potential, a water-conserving effect of kresoxim-methyl has been observed in wheat plants cultivated under vegetation hall conditions (W. Rademacher, Agricultural Center Limburgerhof, personal communication). Relative to corn yield, plants showed reduced water consumption and a lower susceptibility to water stress.

To further elucidate the events leading to kresoxim-methyl-induced retardation of leaf senescence and improved water stress management in wheat, phytohormone levels of ethylene and its biosynthetic precursor ACC (Abeles et al., 1992), indole-3-acetic acid, dihydrozeatin riboside (DZR)- and *trans-zeatin* riboside (ZR)-type cytokinins and (+)-abscisic acid (ABA) were determined in dose-response experiments using senescing leaf discs. Furthermore, the influences of kresoxim-methyl on ABA levels and the water status of plants in hydroponics were investigated.

Materials and Methods

Chemicals

The strobilurin kresoxim-methyl (methyl (*E*)-methoxyimino[-(o-tolyloxy)-o-tolyl] acetate; BAS 490 F; Fig. 1) was from BASF AG, Ludwigshafen, Germany.

Experiments with wheat leaf discs

Discs (0.4 cm in diameter) were cut from leaf blades of wheat (*Triticum aestivum* L. cv. Kanzler) with a corkborer and floated for ca. 1 h in Petri dishes containing double-distilled water so that stress ethylene induced by the excision process could dissipate (Grossmann and Retzlaff, 1997). Twenty randomized discs were placed adaxially on a filter paper in a Petri dish (5 cm in diameter) moistened with 1 mL 10 mmol · L^{-1} MES (2-[N-morpholino]ethanesulfonic acid)-buffer (pH 6.1) containing kresoxim-methyl. Stock solutions of kresoxim-methyl in acetone were diluted 100-fold in the test. Controls were treated only with acetone. In one set of experiments, the leaf discs were incubated in the dark at 25 °C for 20 h. The filter papers with leaf discs were then rolled cylindrically, placed in plastic tubes (13 mm in diameter, 65 mm in height) and sealed with rubber caps. After incubation at 25 °C for a further 5 h, ethylene formation was quantified by gas chromatography (Grossmann and Retzlaff, 1997). In a parallel experiment, leaf discs in Petri dishes were incubated in darkness at 25 °C for 48 h, harvested and powdered under liquid N_2. Total chlorophyll was extracted with cold acetone (0.2 g plant material in 10 mL of 80 % acetone) in triplicate and quantified.

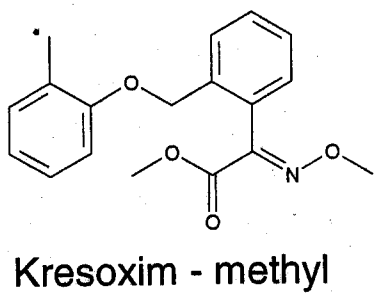

Fig. 1: Structural formula of kresoxim-methyl.

Experiments with wheat plants

Young wheat plants (*Triticum aestivum* L. cv. Kanzler) were raised in vermiculite substrate moistened with ½ strength Linsmaier-Skoog (1964) nutrient solution under controlled conditions (Grossmann and Retzlaff, 1997). Uniformly developed plants that had reached the 2nd leaf stage (approx. 14 cm in height) were transferred to 320 mL glass vessels with ½ strength Linsmaier-Skoog medium and placed in growth chambers with 16/8 h light/dark cycles at 25/20 °C and 75 % relative humidity (9 plants/vessel, 25 replications; light: Osram Powerstar HQI-R 250 W/NDL and Osram Krypton 100 W lamps, photon irradiance ca. 530 µmol m^{-2} s^{-1}, 400–750 nm (Grossmann and Retzlaff, 1997). The solutions were aerated throughout the experiments. After 1 day of adaptation, plants were sprayed uniformly with 0.5 mL/vessel of an aqueous solution containing kresoxim-methyl ($7 \cdot 10^{-4}$ mol · L^{-1}, equivalent to 13 µg/plant, 300 g · ha^{-1} related to the treated area of the glass vessel) prepared in acetone (2 %, w/w, final concentration). In control treatments, aqueous solutions containing corresponding acetone concentrations but without kresoxim-methyl were applied, with no adverse effect on the growth of the plants. To avoid possible kresoxim-methyl effects on untreated plants via the gas phase, controls were cultivated in a separate growth chamber set to give identical environmental conditions. Eight days after treatment, growth parameters were measured and shoots and roots from replicate vessels were harvested, immediately frozen in solid CO_2 and stored at −80 °C. The amount of transpired water was monitored by weighing the vessels. The values were referred to shoot dry weight. Stomatal behaviour in the primary leaves of plants in parallel vessels was determined by measuring diffusive resistance to water vapour transport with a diffusion porometer (LI-65 LI-COR Autoporometer, Bachofer, Reutlingen, Germany) with a horizontal sensor (LI-20S). The values of diffusion resistance were the means of 30 replications, which were calculated as a percentage of the control.

Determination of ACC

Samples of powdered plant material (100 mg, 3 replications) were extracted with 70 % aqueous ethanol. The ACC content was assayed following conversion to ethylene (Lizada and Yang, 1979), by gas chromatography (Grossmann and Retzlaff, 1997).

Extraction and determination of phytohormones

Powdered plant material (ca. 0.8 g) was extracted with 80 % methanol (3 replicate extractions) and the extracts were passed through a C_{18}-reversed phase prepacked column (SEPPAK; Waters, Königstein, Germany) as described (Weiler et al., 1986; Grossmann and Retzlaff, 1997). The effluent was concentrated *in vacuo* and dissolved in 1.8 mL 5 % aqueous methanol containing 0.1 mol · L^{-1} acetic acid (solution A). An aliquot of the extract (1.0 mL) was separated by high performance liquid chromatography (HPLC) on a reverse-phase Nucleosil 120–5 µm C_{18} column using a linear gradient from solution A to 95 % methanol. The gradient sweep time was 30 min at a flow rate of 2.8 mL · min^{-1}. The fractions containing ZR- and DZR-type cytokinins (15.0 min, including *trans-zeatin* and dihydrozeatin), IAA (20.0 min), and ABA (22.3 min) were collected and the quantitative determination was performed by enzyme-immunoassay with monoclonal antibodies 100 % reactive against

their respective antigens (Weiler et al., 1986). IAA content was assayed after methylation with ethereal diazomethane to IAA methyl ester. The antibodies were kindly provided by Professor E. W. Weiler (University of Bochum, Germany). The detection limit was ca. 1 pmol for IAA and 0.1 pmol for all other phytohormones, as estimated from standard curves. All samples were assayed at least in triplicate and the concentrations were expressed as the equivalents of phytohormone in pmol/g fresh weight. Internal performance controls of assay accuracy and reliability were carried out as described (Weiler et al., 1986; Grossmann and Retzlaff, 1997).

All experiments were repeated at least twice and proved to be reproducible. The results of a representative experiment are shown.

Results and Discussion

Delayed senescence following kresoxim-methyl treatment

Two days after treatment of wheat leaf discs with kresoxim-methyl, chlorophyll loss as a parameter for the progress in senescence was reduced with increasing compound concentrations (Fig. 2). Maximum delay in senescence of the leaf discs with an up to 83 % higher level of total chlorophyll compared with controls was obtained at $3 \cdot 10^{-4}$ mol · L^{-1} kresoxim-methyl. The dose-response curve of elevated total chlorophyll after kresoxim-methyl treatment was closely correlated with decreased levels of ACC and ethylene formation and a rise in DZR-type cytokinins (Fig. 2). While ethylene synthesis was inhibited by up to 60 % at 10^{-4} mol · L^{-1} kresoxim-methyl, the levels of DZR-type cytokinins increased up to 3-fold. Concentration-dependent increases to a maximum of 223 % of the control were also observed in endogenous ABA levels. In contrast, the levels of immunoreactive ZR-type cytokinins and IAA did not change significantly (Fig. 2).

This suggests that the auxin-like activity of kresoxim-methyl, which was characterized in several biotests (Grossmann and Retzlaff, 1997), appears not to be mediated through an influence on the auxin status *in planta*. Kresoxim-methyl inhibited ACC and ethylene synthesis, which was causally linked with delayed leaf senescence (Grossmann and Retzlaff, 1997). Kresoxim-methyl affected the induction process of ACC synthase activity (Grossmann and Retzlaff, 1997). Since ethylene has been shown to decrease endogenous levels of cytokinins, possibly by accelerating their degradation (Bollmark and Eliasson, 1990; Grossmann et al., 1993), reduced levels of ethylene should therefore result in maintained or increased cytokinin contents. Cytokinins play a major role in the delay of leaf senescence (Nooden and Leopold, 1988; Grossmann et al., 1991; Nam, 1997), whereas ethylene accelerates this process (Abeles et al., 1992; Nam, 1997). In wheat leaf discs, kresoxim-methyl shifted the hormonal balance, favouring particularly the DZR-type cytokinins as opposed to ethylene (Fig. 2). In close correlation, leaf senescence was retarded. Similar hormonal changes in shoot tissue were observed when wheat plants at the 2nd leaf stage were foliar-treated with kresoxim-methyl for 48 h (Grossmann and Retzlaff, 1997). However, the levels of ABA-like material in the shoot tissue were elevated only slightly (Grossmann and Retzlaff, 1997). In contrast, in the experiments using wheat leaf discs treated with kresoxim-methyl, endogenous ABA levels increased 2-fold, relative to the controls (Fig. 2). Second to ethylene, ABA is usually considered to be a

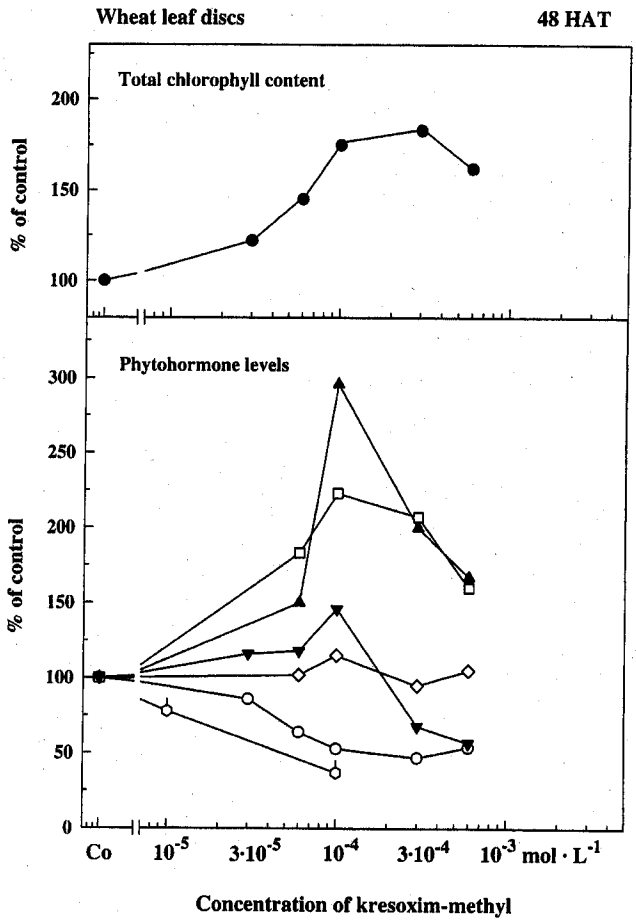

Fig. 2: Influence of kresoxim-methyl on total chlorophyll content, ethylene formation and immunoreactive phytohormone levels in senescing leaf discs of wheat after 48 h of treatment in the dark. Control values ± SE representing 100 % levels of phytohormone-like material (pmol equivalents · g^{-1} fresh weight) were: 230 ± 6 (ethylene formation, ○), 3016 ± 200 (ACC, ○), 1.26 ± 0.05 (ZR, ▼), 1.26 ± 0.15 (DZR, ▲), 2905 ± 104 (IAA, ◇), 4.91 ± 0.68 (ABA, □). Control values ± SE of total chlorophyll content were 488 ± 36 µg · g^{-1} fresh weight.

senescence-promoting hormone, although its endogenous function is not clear (Nooden and Leopold, 1988). However, after manipulation of endogenous ABA levels by auxin herbicides and IAA at high concentrations (Grossmann and Scheltrup, 1995; Grossmann et al., 1996) or when ABA was applied exogenously (Grossmann and Jung, 1982; Grossmann and Scheltrup, 1995), promotion of senescence could only be achieved at rather high endogenous ABA concentrations.

Water-conserving effects following kresoxim-methyl treatment

Clear evidence exists that ABA is involved in plant responses to water deficit (Hartung and Davies, 1991). ABA improves the plants water status, particularly by reducing stomatal aperture and thus transpiration (Hartung and Davies, 1991). Recently, kresoxim-methyl was shown to increase water-use efficiency in wheat (W. Rademacher, Agricultural

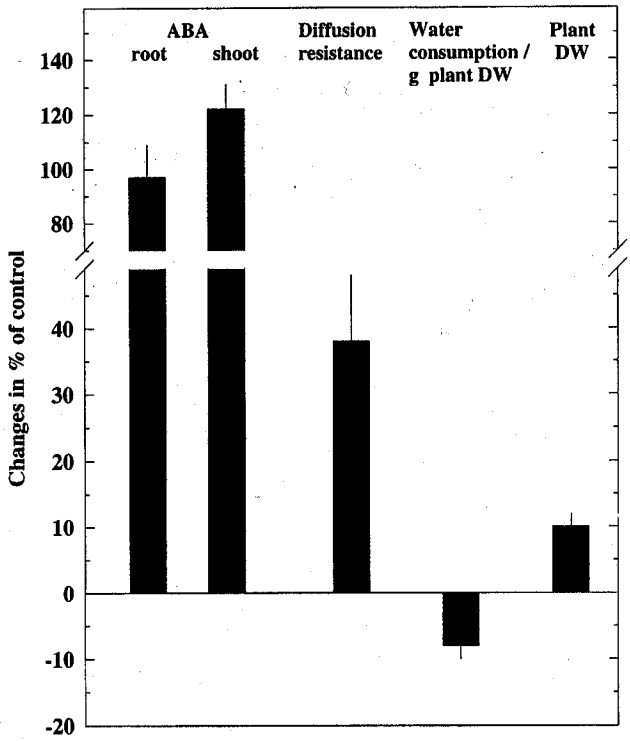

Fig. 3: Changes in immunoreactive ABA levels in root and shoot tissue, leaf diffusion resistance, water consumption and plant dry weight (DW) after foliar treatment of wheat plants at the 2nd leaf stage in hydroponics with kresoxim-methyl for 8 days. Vertical bars represent SE of the means, which are significantly different to control at $P < 0.05$ (water consumption, plant dry weight) or $P < 0.01$ (ABA levels, leaf diffusion resistance) when statistically analysed using a t-test. Control values ± SE representing 0 % change were: 101 ± 3 (shoot) and 187 ± 40 (root) pmol ABA equivalents · g^{-1} dry weight, 102.6 ± 6.6 s · cm^{-1} (diffusive resistance), 281 ± 6 mL · g^{-1} plant dry weight (water consumption), 94 ± 2 mg (plant dry weight).

Center Limburgerhof, personal communication). In order to obtain a clearer picture of whether kresoxim-methyl is able to limit the water consumption of wheat plants through increases in endogenous ABA levels and induction of stomatal closure, long-term experiments were performed. Wheat plants at the 2nd leaf stage in hydroponics were foliar-treated with $7 \cdot 10^{-4}$ mol · L^{-1} (equivalent to 300 g ha^{-1}) kresoxim-methyl for 8 days. A 2-fold stimulation of ABA levels was found in shoot and root tissue which coincided with a reduction in stomatal aperture, as determined by increased water vapour diffusive resistance (Fig. 3). After exogenous exposure of wheat plants to 10^{-7} mol · L^{-1} ABA via the root, the ABA level in the shoot tissue corresponded quantitatively with a similar reduction in stomatal aperture found after kresoxim-methyl treatment (data not shown). Concomitantly, based on plant dry weight, water consumption was reduced by 8 % in kresoxim-methyl-treated plants in comparison to control (Fig. 3). In addition, the dry weight of plants increased by 10 % (shoot dry weight by 9 %, root dry weight by 13 %). The latter effect could be explained by the higher CO_2 assimilation observed under the influence of kresoxim-methyl (Grossmann and Retzlaff, 1997). The reduced stomatal aperture appears to be not limiting for CO_2 uptake. It is assumed that the regulation of stomatal aperture, mediated by kresoxim-methyl-induced ABA, results in reduced water loss by transpiration. This effect of kresoxim-methyl could improve the water status and stress management of wheat plants under water deficit conditions.

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