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# Bioregulatory Effects of the Fungicidal Strobilurin Kresoxim-methyl in Wheat (*Triticum aestivum*)

Klaus Grossmann* & Günter Retzlaff

BASF Agricultural Research Station, D-67114 Limburgerhof, Germany

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**Abstract:** Apart from its fungicidal effect, the strobilurin kresoxim-methyl (BAS 490 F) was found to induce physiological and developmental alterations in wheat (*Triticum aestivum* L.) which are seen in connection with improved yield. In a series of biotests including heterotrophic maize and photoautotrophic algal cell suspensions, duckweed, isolated mustard shoots and germinating cress seeds, kresoxim-methyl showed a similar response pattern to standard auxins (e.g. indol-3-ylacetic acid, IAA; 2-(1-naphthyl)acetic acid, α-NAA). Auxin-like activity of kresoxim-methyl was also found when stem explants of tobacco were cultured on a hormone-free medium. Kresoxim-methyl stimulated shoot formation, particularly at $10^{-7}$ M. The same effect was induced by $10^{-8}$ M IAA. The determination of phytohormone-like substances in shoots of wheat plants foliar-treated with $7 \times 10^{-4}$ M kresoxim-methyl revealed only slightly changed levels of endogenous IAA, gibberellins and abscisic acid. In contrast, the contents of dihydrozeatin riboside-type cytokinins increased to 160% of the control, while *trans*-zeatin riboside- and isopentenyladenosine-type cytokinins remained nearly unchanged. The most remarkable alterations were the reductions in 1-aminocyclopropane-1-carboxylic acid (ACC) levels and ethylene formation which were demonstrated in intact plants, leaf discs and the shoots of wheat subjected to drought stress. Kresoxim-methyl affected the induction of ACC synthase activity which converts *S*-adenosyl-methionine to ACC in ethylene biosynthesis. In shoots from foliar-treated wheat plants, $10^{-4}$ M kresoxim-methyl inhibited stress-induced increases in endogenous ACC synthase activity, ACC levels and ethylene formation by approximately 50%. Reductions in ACC synthase activity and ACC levels of 30% were also obtained at low concentrations of α-NAA ($10^{-6}$ M). In contrast, ACC synthase activity *in vitro* was not influenced by adding the compounds. In wheat leaf discs, the inhibiting effect of kresoxim-methyl, α-NAA and IAA on ethylene formation was accompanied by delayed leaf senescence, characterized by reduced chlorophyll loss. However, in contrast to kresoxim-methyl which showed only inhibitory activity on ethylene synthesis over a wide range of concentrations applied, the auxins stimulated ethylene production at high concentrations of about $10^{-4}$ M. The inhibition of ethylene biosynthesis by kresoxim-methyl, together with an increase in endogenous cytokinins could explain the retardation of senescence and the intensified green leaf pigmentation in wheat exposed to this strobilurin.

Key words: 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, auxin activity, cytokinins, ethylene biosynthesis, kresoxim-methyl, leaf senescence

## 1 INTRODUCTION

Kresoxim-methyl (BAS 490 F) belongs to a new class of fungicides which are derived from the fungal secondary metabolite strobilurin.[1,2] The compound is characterized by its broad-spectrum fungicidal activity against numerous foliar pathogens from the ascomycetes, basidiomycetes, deuteromycetes and oomycetes in e.g. pome fruits, grape vine, vegetables and cereals. The uptake and translocation properties provide protective,

* To whom correspondence should be addressed.



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curative, eradicative and long residual disease control.[3] The mode of action is the effective inhibition of mitochondrial respiration by blocking electron transfer at the cytochrome-bc$_1$ complex. The binding site is the ubihydroquinone cytochrome-c oxidoreductase.[1,2,4] Studies using mitochondrial preparations from yeast, *Botrytis cinerea*, house fly, rat and maize revealed little variation in enzymatic sensitivity to strobilurins at the mitochondrial target level.[2] The biokinetic properties of strobilurins (uptake, transport, metabolism) are decisive for species selectivity.[2]

In addition to its direct effect on the fungus, kresoxim-methyl has been found to induce physiological alterations in various crops which are often seen in connection with a positive influence on yield.[5] In wheat, these non-fungicidal, secondary effects include a darker green appearance of the leaves, retardation of senescence with enhanced concentrations of chlorophyll and protein and increased biomass production with favoured harvest index.[5] Kresoxim-methyl significantly changed the carbon dioxide compensation point of wheat plants in favour of an improved photosynthetic carbon dioxide uptake as opposed to the respiratory carbon dioxide release.[6] This effect could be explained by a transient inhibition of plant respiration which was observed in wheat plants after foliar treatment with kresoxim-methyl (unpublished data). The compound did not alter the photosynthetic electron transport in photosystem II, as measured in the Hill reaction with isolated wheat thylakoids (unpublished data). In consequence, an increase in shoot growth and thus in the photosynthetically active green tissues was observed (unpublished results) which led to further increases in the photosynthetic capacity of treated plants. However, these interactions are not sufficient to account for the all physiological and developmental effects elicited by the compound. Particularly, the delay in senescence caused by kresoxim-methyl in wheat can kindle speculations as to whether changes in the hormone status of the plant may be implicated in the underlying mode of action. Possible effects of kresoxim-methyl on phytohormonal metabolism or signalling as well as a bioregulatory 'hormone-like' activity of the compound itself might play a role.

In the present work a set of selected biotests using maize cell suspensions, algae, duckweed, isolated mustard shoots and germinating cress seeds was used to obtain a physiological fingerprint of kresoxim-methyl. The response pattern was compared with profiles of compounds with known modes of action to classify the bioregulatory activity of kresoxim-methyl. Since similarities to auxins were found, kresoxim-methyl was further characterized in bioassays for auxin-like activity. In addition, phytohormone levels of indol-3-ylacetic acid (IAA), gibberellins, abscisic acid (ABA), cytokinins and ethylene were determined in wheat plants, following foliar treatment with the compound. The effects, particularly on 1-aminocyclopropane-1-carboxylic acid (ACC) production in ethylene biosynthesis, were characterized and compared with the induced physiological alterations in biomass production and senescence.

## 2 MATERIALS AND METHODS

### 2.1 Chemicals

The fungicide kresoxim-methyl (methyl (*E*)-methoxyimino-α-(*o*-tolyloxy)-*o*-tolylacetate; BAS 490 F, Fig. 1) was from BASF AG, Ludwigshafen, Germany. 2-Chloroethylphosphonic acid (ethephon) was obtained from Calbiochem (Frankfurt, Germany), aminoethoxyvinylglycine (AVG) from Fluka (Neu-Ulm, Germany), indol-3-ylacetic acid (IAA), 2-(1-naphthyl)acetic acid (α-NAA), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) from Riedel-de Haen (Seelze, Germany). The other compounds, including 6-benzylaminopurine (BAP) were obtained from Sigma (München, Germany). Stock solutions of the chemical compounds in acetone (kresoxim-methyl, IAA, α-NAA, MCPA, 2,4-D) or in water (AVG, ethephon) were diluted 100-fold in the test.

### 2.2 Bioassays

#### 2.2.1 *Maize cell suspension bioassay*

Freely suspended callus cells from maize (*Zea mays* L. cv. Black Mexican Sweet) were cultivated in a modified Murashige–Skoog medium and the bioassay was performed as described previously.[7] The cells were subcultured at seven-day intervals. Acetone solutions of the compounds were pipetted into plastic tubes and the solvent allowed to evaporate before adding 2 ml of exponentially growing cell suspension. The tubes (three replicates) were shaken at 400 rev min$^{-1}$ and 25°C in the dark on a rotary shaker. After incubation for eight days, the conductivity of the medium was measured as the growth parameter.

#### 2.2.2 *Algae bioassay*

Cells of *Scenedesmus acutus* (culture collection Göttingen, 276-3a) were propagated photoautotrophically.[7]

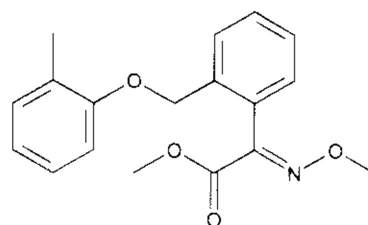


**Fig. 1.** Structural formula of kresoxim-methyl.

The bioassay was carried out in plastic microtitre dishes containing 24 wells.[7] Before loading the wells with 0·5 ml cell suspension each (two replicates), 0·5 ml medium and compound in acetone solution were added, allowing the organic solvent to volatilize. The 15 additional compartments between the wells were filled with sodium carbonate/bicarbonate buffer. The dishes were sealed with plastic lids and incubated on a shaker under continuous light at 23°C. After 24 h, the contents of each well were removed and cell numbers were determined by a Coulter Counter.

*2.2.3 Lemna bioassay*

Stock cultures of *Lemna paucicostata* L. were propagated mixotrophically in an inorganic medium containing sucrose.[7] The bioassay was conducted under aseptic conditions in plastic Petri dishes (5 cm in diameter).[7] The test compounds were added to the dishes in acetone solution and the organic solvent allowed to volatilize before loading them with 15 ml medium without sucrose followed by four fronds each. The culture dishes were then closed with plastic lids and incubated under continuous light (Philips TL white neon tubes, *c*.40 $\mu$mol m$^{-2}$ s$^{-1}$, 400–750 nm) in a growth chamber at 25°C. Eight days after treatment, the increase of the area covered by the fronds in each dish was determined as the growth parameter using an image analysing system.[7] The area of fronds before incubation was subtracted from this value.

*2.2.4 Isolated shoot bioassay*

Seedlings of mustard (*Sinapis alba* L.) were grown under standardized greenhouse conditions. The shoots were removed, weighed and placed upright in plastic vials (25 mm in diameter, 38 mm in height; Greiner, Nürtingen, Germany) containing 12 ml double-distilled water and the test compound added in acetone solution. To avoid evaporation, the vials were closed with plastic covers with slits into which the shoots were fitted (three shoots per vial). The vials were cultivated in growth chambers with 16/8 h light/dark cycles at 24/22°C (light: Osram krypton 100-W lamps and Osram universal white neon tubes, *c*.200 $\mu$mol m$^{-2}$ s$^{-1}$, 400–750 nm). After three days the changes in fresh weight were measured by weighing the shoots and subtracting the value from the weight at the beginning.

*2.2.5 Cress germination bioassay*

Seeds of cress (*Lepidium sativum* L.) were placed in plastic Petri dishes (5 cm in diameter) filled with a vermiculite substrate. Stock solutions of the test compounds in acetone were added, together with 8 ml water.[7] Control seeds were moistened only with water and acetone. The dishes were incubated in a growth chamber at 25°C in the dark until the onset of germination (approximately two days). Then, they were kept for five days at room temperature and normal day/night conditions in the laboratory. Inhibition of germination was evaluated visually (0 = no influence, 100 = total inhibition).

The results were expressed as percentage inhibition or reduction relative to untreated controls. Mean values of three replicates are given as the percentage growth inhibition relative to the control. Individual standard errors were less than 5%.

## 2.3 Experiments with tobacco stem explants

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were raised on a nutrient-supplemented peat-based substrate under controlled conditions. Pieces of stem 15 cm in length were excised from the stem approximately 20 cm below the shoot apex of plants in flower (80 cm in height). The leaves and lateral buds were removed and the stem pieces were cut into 2-cm segments which were surface-sterilized with 70% ethanol for 2 min and with 6% sodium hypochlorite for 10 min. Afterwards, the outer layers of tissue were peeled off and the segments were cut transversely into discs, each about 2 mm thick. The stem discs were halved and transferred to sterilized cylindrical plastic containers (65 mm in diameter, 65 mm in height; Greiner, Nürtingen, Germany; three discs per container, three replicates) with 65 ml modified Murashige–Skoog medium with amino acids and vitamins[7] and the test compound (in acetone or water solution) solidified with agar (1·1%). The containers were sealed with plastic covers and incubated for 10 weeks under dim light at 25°C.

## 2.4 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 cork-borer and floated for *c*.1 h in Petri dishes containing double-distilled water so that stress ethylene induced by the excision process could dissipate.[8] Twenty randomized discs were placed adaxially on a filter paper in a Petri dish (5 cm in diameter) moistened with 1 ml MES (2-[*N*-morpholino]ethanesulfonic acid) buffer (pH 6·1; 10 mM) containing the test compounds. In one set of experiments, the leaf discs were incubated in continuous light (*c*.55 $\mu$mol m$^{-2}$ s$^{-1}$, 400–750 nm, Osram universal white neon tubes) 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 in light at 25°C for 5 h, ethylene formation was quantified by gas chromatography.[8] In a parallel experiment, leaf discs in Petri dishes were incubated in darkness at 25°C for three days, harvested and powdered under liquid nitrogen. Total chlorophyll was extracted with cold

acetone (0·2 g plant material in 10 ml of 80% acetone) in triplicate and quantified.[9]

### 2.5 Experiments with wheat plants

Young wheat plants (*Triticum aestivum* L. cv. Kanzler) were raised in vermiculite substrate moistened with half-strength Linsmaier–Skoog[10] nutrient solution under controlled conditions.[11] Uniformly developed plants which had reached the 2nd-leaf stage (approx. 14 cm in height) were transferred to 320-ml glass vessels with half-strength Linsmaier–Skoog medium and placed in growth chambers with 16/8 h light/dark cycles at 25/20°C and 75% relative humidity (nine plants per vessel, five replications; light: Osram Powerstar HQI-R 250 W/NDL and Osram Krypton 100-W lamps, *c*.530 $\mu$mol m$^{-2}$ s$^{-1}$, 400–750 nm.[11] The solutions were aerated throughout the experiments. After one day of adaptation, plants were sprayed uniformly with 0·5 ml per vessel of an aqueous solution containing kresoxim-methyl ($7 \times 10^{-4}$ M, equivalent to 13 $\mu$g per plant, 300 g ha$^{-1}$ related to the treated area of the glass vessel) prepared in acetone (16 g litre$^{-1}$ 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. At various times after treatment, growth parameters were measured and shoots and roots from replicate vessels were harvested, immediately frozen in solid carbon dioxide and stored at −80°C.

In a further set of experiments, wheat plants were raised in vermiculite substrate and groups of plants were directly sprayed with kresoxim-methyl or α-NAA (0·5 ml on 80 plants). After 3 h incubation in growth chambers under the conditions described above, water stress was initiated by incubating detached shoots at 22°C and 50% relative humidity. After 1 h, shoots were harvested, immediately frozen in solid carbon dioxide and stored at −80°C. For determination of ethylene formation, detached and stressed shoots were subsequently incubated in glass tubes (17 mm in diameter, 200 mm in height; three shoots per tube) which were sealed with rubber caps. After incubation in light at 25°C for 2 h, ethylene formation was quantified by gas chromatography.[8]

### 2.6 Analytical determinations

#### *2.6.1 Determination of ACC and ethylene*

Plant material was powdered under liquid nitrogen and samples (100 mg, three replications) were extracted with 70% aqueous ethanol. The ACC content was assayed, following conversion to ethylene,[12] by gas chromatography.[8]

#### *2.6.2 Determination of ACC synthase activity*

ACC synthase (EC 4.4.1.4) was extracted and assayed as described previously.[13] Powdered plant material (1·5 g; two replicates) was homogenized in 100 mM EPPS (*N*-[2-hydroxyethyl]piperazine-*N*′-3-propanesulfonic acid)/KOH buffer (pH 8·5) containing dithiothreitol (5 mM), pyridoxal phosphate (6 $\mu$M), leupeptin (10 $\mu$M) and Pefabloc SC (4-(2-aminoethyl)phenylsulfonylfluoride hydrochloride, Merck, Darmstadt, Germany; 10 $\mu$M). The extract was centrifuged and the supernatant was passed through a Sephadex G 25 column (Pharmacia, Uppsala, Sweden) which had been equilibrated with EPPS buffer (5 mM; pH 8·5) containing dithiothreitol (1 mM), pyridoxal phosphate (6 $\mu$M) and Pefabloc SC (10 $\mu$M). The ACC synthase assay mixture, with a total volume of 0·6 ml, contained 0·3 ml enzyme preparation in 80 mM EPPS buffer with 20 $\mu$M pyridoxal phosphate and 100 $\mu$M *S*-adenosyl methionine. After an incubation period of 2 h at 37°C, the reaction was stopped by adding 20 $\mu$mol mercury (II) chloride. Subsequently, the ACC produced was determined by chemical conversion to ethylene.[12] All assays were performed with four replicates. Protein was determined in the enzyme extracts[14] using the Bio-Rad protein assay reagent (München, Germany) with bovine serum albumin as a standard. For investigation of kresoxim-methyl effects on ACC synthase activity *in vitro*, ACC synthase was extracted from water-stressed shoot material and 60 $\mu$l of an aqueous solution containing kresoxim-methyl prepared as a stock solution in dimethyl sulfoxide (50 g litre$^{-1}$ final concentration in the assay) was added to the assay mixture before incubation.

#### *2.6.3 Extraction and determination of phytohormones*

Powdered plant material (1 g) was extracted with 80% methanol (three replicate extractions) and the extracts were passed through a $C_{18}$-reversed phase prepacked column (SEPPAK; Waters, Königstein, Germany) as described.[15,16] The effluent was concentrated under vacuum and dissolved in double-distilled water (3 ml). *Trans*-zeatin riboside (ZR)-, dihydrozeatin riboside (DZR)- and isopentenyladenosine (iPA)-type cytokinins and (+)-ABA in an aliquot of the extract (1·5 ml) were separated by high performance liquid chromatography (HPLC) on a reverse-phase Nucleosil 120-5 $\mu$m $C_{18}$ column using a linear gradient from methanol + water (5 + 95 by volume) containing 0·1 M acetic acid (solution A) to methanol + water (95 + 5 by volume) (solution B). The fractions containing the phytohormones were collected and the quantitative determination was performed by enzyme-immunoassay with monoclonal antibodies 100% reactive against their respective antigens.[15] For determination of gibberellins

(GAs) and IAA, the residual 1·5 ml of the effluent from the SEPPAK column was acidified with 1 M hydrochloric acid, and extracted with ethyl acetate (3 × 3 ml). After concentration to dryness, the extract was redissolved in methanol (200 $\mu$l) and methylated with ethereal diazomethane.[16] IAA methyl ester content was directly assayed by enzyme-immunoassay, while GAs were quantified by radioimmunoassay with a polyclonal antiserum against $GA_1$ methyl ester.[15] The antibodies were kindly provided by Professor E. W. Weiler (University of Bochum, Germany). The detection limit was *c*.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 $g^{-1}$ fresh weight. Internal performance controls of assay accuracy and reliability were carried out as described.[15,16]

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

## 3 RESULTS AND DISCUSSION

### 3.1 Physiological profiling using bioassays

Recently, we have developed a set of five bioassays using heterotrophic maize and photoautotrophic green algae (*Scenedesmus acutus*) cell suspensions, duckweed (*Lemna paucicostata*), isolated mustard shoots and germinating cress seeds which are useful to characterize and classify the physiological mode of action of new compounds in the search for their biochemical targets.[7] The response pattern, which includes influences on germination and the vegetative growth of cells and organized tissues, represents a physiological fingerprint of a compound. The results can be interpreted directly or a library of response patterns of compounds with known mode of action can be screened for similarities. Although this is not an absolutely conclusive way of testing for any specific mode of action, it does provide some clues which can be used as an aid to direct further investigations. At the high concentrations applied kresoxim-methyl inhibited growth of maize cell suspensions, *Lemna* and germinating cress seeds (Fig. 2). No preference either for the heterotrophic (maize cell suspension, germination of cress seeds) or photoautotrophic (algae, *Lemna*, mustard shoot) systems was observed. Green algae and isolated mustard shoots showed only slight response. In *Lemna* growth inhibition was accompanied by an intensified chlorophyll pigmentation. Nearly the same type of physiological profile in the various systems was obtained with natural (IAA) and synthetic auxins (α-NAA) and auxin herbicides, such as MCPA and 2,4-D (Fig. 2). Maize cell suspensions, *Lemna* and germinating cress seeds were highly affected, whereas green algae and mustard shoots showed only

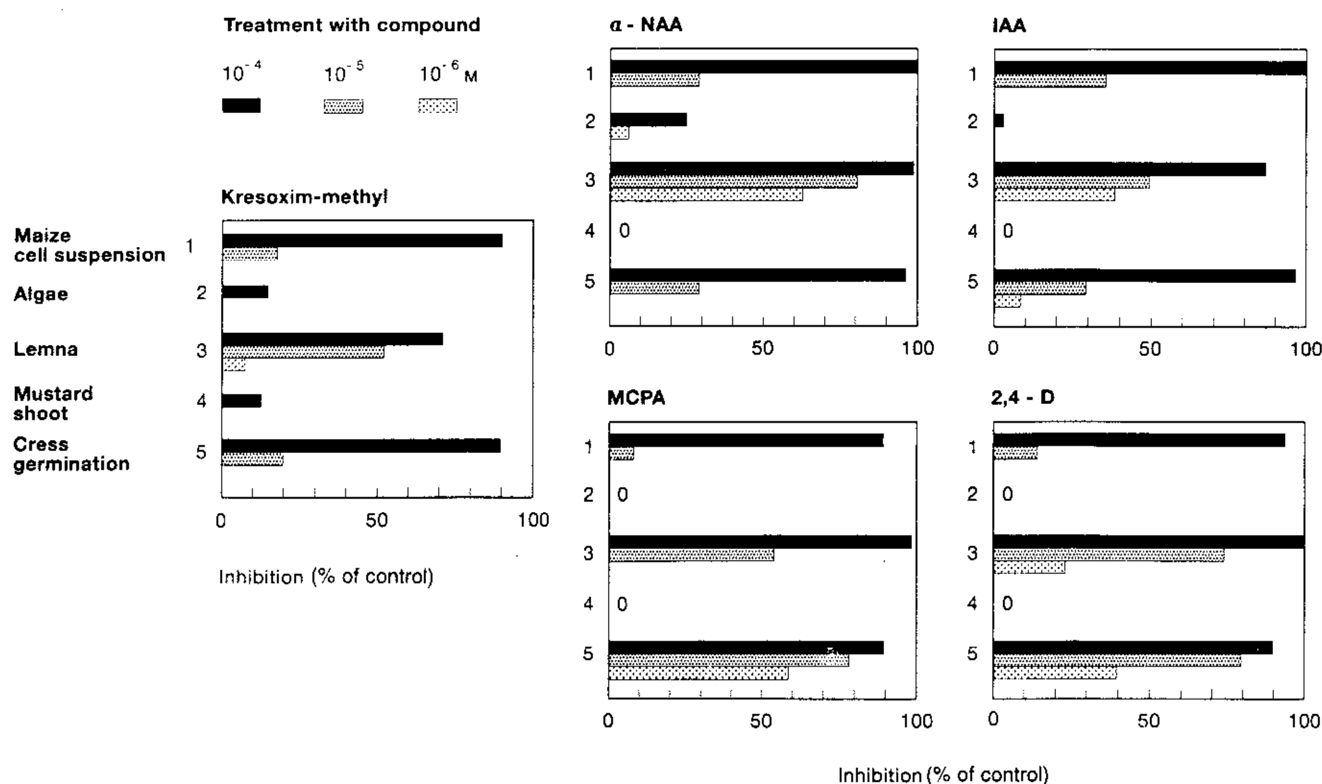


**Fig. 2.** Effects of kresoxim-methyl 2-(1-naphthyl)acetic acid (α-NAA), indol-3-yl acetic acid (IAA), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in biotests including maize and algal cell suspensions, duckweed, isolated mustard shoots and germinating cress seeds. SE of the mean in all cases less than 5%.

low susceptibility. However, in contrast to kresoxim-methyl, the auxin compounds induced epinastic deformation of cress seedlings. Auxins are known to regulate plant growth and development by influencing fundamental processes, such as cell elongation and division.[17] However, their effects on growth are extremely diverse and biphasic. Dependent on the concentration and biological activity, opposite effects are observed.[18] With regard to its physiological profile, it is suggested that kresoxim-methyl might possess auxin-like activity. In accordance with this conclusion, kresoxim-methyl was able to alleviate growth inhibition in *Lemna* caused by $10^{-5}$ M BAP (Fig. 3). This anticytokinin effect was also observed after the application of auxins, such as α-NAA (Fig. 3). Furthermore, when stem explants of tobacco were cultured *in vitro* on a medium containing kresoxim-methyl, but no phytohormones, morphogenesis of shoots and subsequently roots was induced, particularly at concentrations of about $10^{-7}$ M (Fig. 4). In the control explants only proliferation of callus was observed. Explants grown on $10^{-4}$ to $10^{-6}$ M IAA produced exclusively callus (not shown). At concentrations of $10^{-8}$ M IAA, shoot initiation was induced (not shown). However, it should be mentioned that the stem explants used in this study included vascular tissue which could supply phytohormones such as cytokinins endogenously[19] which might have influenced the response of the explant tissue to the exogenously applied compounds.

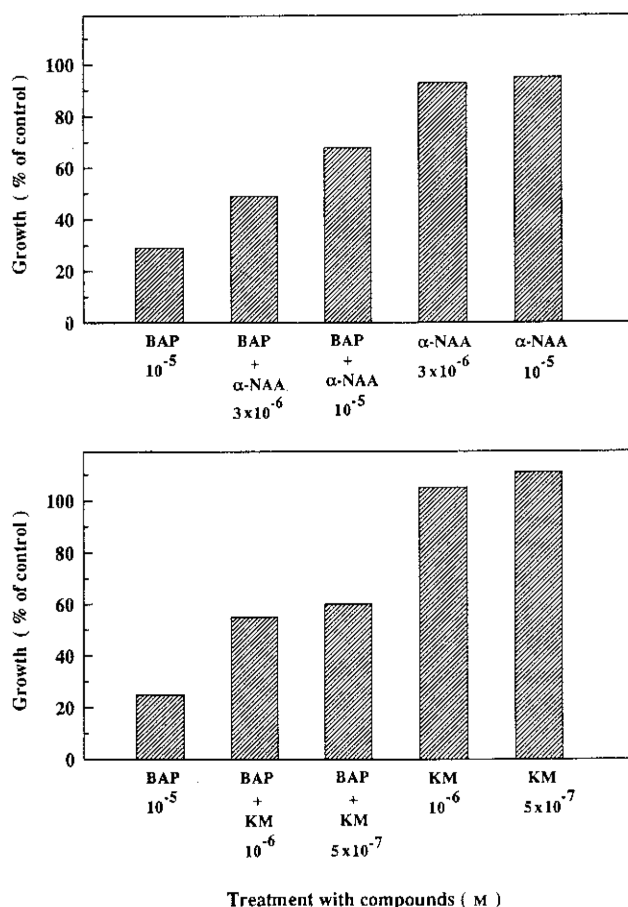


**Fig. 3.** Influence of kresoxim-methyl (KM) and 2-(1-naphthyl) acetic acid (α-NAA) on 6-benzylaminopurine (BAP)-induced inhibition of growth in *Lemna paucicostata*. SE of the mean in all cases less than 5%.

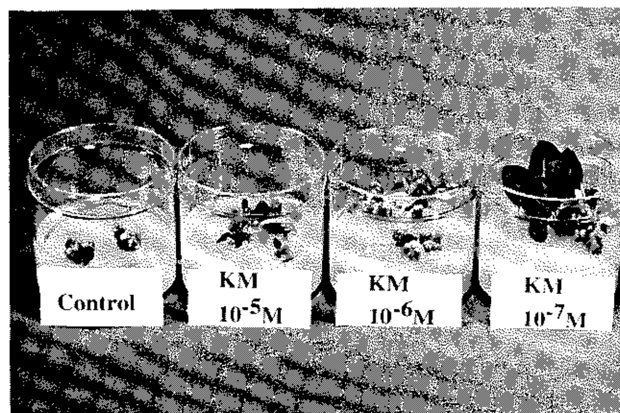


**Fig. 4.** Influence of kresoxim-methyl (KM) on organ formation in stem explants of tobacco after 10 weeks of incubation on agar-solidified medium.

## 3.2 Phytohormonal changes in wheat plants

In order to obtain a clearer picture of whether kresoxim-methyl itself has auxin-like activity or the compound is able to change the auxin status *in planta*, the effects of the compound on the contents of IAA and other groups of phytohormones, including immunoreactive gibberellins, cytokinins and ABA in shoots of wheat plants were studied. In addition, the contents of 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene in its biosynthesis,[20] were determined. After foliar treatment with $7\times10^{-4}$ M kresoxim-methyl for 48 h, the levels of IAA, $GA_1$- and ABA-like material were slightly increased in the shoot tissue (Fig. 5). Levels of ZR- and particularly DZR-type cytokinins in the shoot increased to 113% and 160% of the control, respectively, while iPA-type cytokinins were reduced, though not significantly. Concomitantly, a reduction of nearly 30% in ACC levels, relative to controls, was observed (Fig. 5). When plants were incubated for a further six days after treatment with kresoxim-methyl, a 12% increase in plant fresh weight compared to controls was found.

In conclusion, kresoxim-methyl did not markedly stimulate endogenous auxin levels. On the contrary, when determined 24 h after foliar treatment, even decreased levels of IAA in the shoot tissue were observed (data not shown). The compound increased particularly the amount of immunoreactive DZR-type cytokinins in the shoot tissue, whereas ACC levels declined. The latter effect might be explained by an inhibitory influence of kresoxim-methyl on a step prior to the conversion of ACC to ethylene in its biosynthetic pathway. In accordance with this hypothesis, kresoxim-

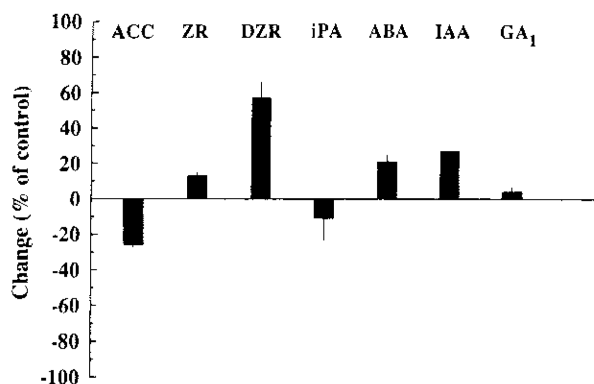


**Fig. 5.** Changes in immunoreactive phytohormone levels and 1-aminocyclopropane-1-carboxylic acid (ACC) contents in shoots of wheat plants after foliar treatment with kresoxim-methyl for 48 h. Control values (±SE) representing 100% levels of phytohormone-like material (pmol equivalent $g^{-1}$ fresh weight) were: ACC, 1920(±200); ZR, *trans*-zeatin riboside, 8(±1); DZR, dihydrozeatin riboside, 7(±1); iPA, isopentenyladenosine, 102(±4); ABA, (+)-abscisic acid, 19(±2); IAA, indol-3-ylacetic acid (1052 ± 21); $GA_1$, gibberellin $A_1$, 28(±1).

methyl was shown to decrease ACC levels and ethylene formation by 46% and 26%, respectively, in heterotrophic cell suspension cultures of sunflower after treatment with $10^{-5}$ M of the compound for seven days (F. Siefert and K. Grossmann, unpublished data).

### 3.3 Influence on ACC synthase activity

The key enzyme in ethylene biosynthesis is ACC synthase which catalyses the conversion of *S*-adenosylmethionine to ACC.[20] Auxins have been shown to regulate *de-novo* synthesis of this enzyme.[20] Since the levels of ACC synthase activity in young wheat plants were very low, we stress-induced the enzymatic activity in order to evaluate more precisely a possible influence of kresoxim-methyl on ACC synthase activity *in vivo*. First, plants were foliar-treated with increasing concentrations of kresoxim-methyl for 3 h. Subsequently, water stress was initiated by allowing detached shoots to lose fresh weight under reduced humidity conditions. During 1 h of stress, the fresh weight of detached shoots decreased by approximately 12%, while ACC synthase activity increased about 80-fold relative to intact, non-stressed shoots. Dependent on the concentration, kresoxim-methyl effectively reduced ACC synthase activity and ACC levels in the tissue by 50% at $10^{-4}$ M (Fig. 6). Under these conditions, ethylene formation was inhibited by 36% (from 1·43 to 0·92 nmol $g^{-1}$ FW $h^{-1}$). Foliar treatment of wheat plants with α-NAA also decreased this enzymatic activity (Fig. 6). Reductions of up to 30% were obtained at low concentrations of about $10^{-6}$ M. Similar changes in ACC synthase activity per mg protein were induced (not shown). Closely correlated with the reduced enzyme activity, ACC levels in the tissue declined below those of controls (Fig. 6). In contrast, additions of

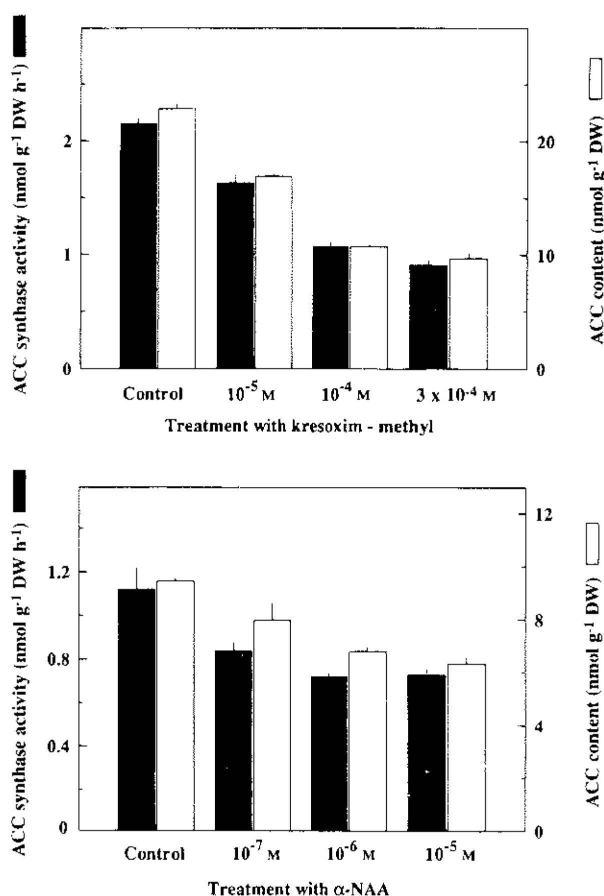


**Fig. 6.** Effects of kresoxim-methyl and 2-(1-naphthyl)acetic acid (α-NAA) on 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity and ACC levels in shoots from wheat. Wheat plants were foliar-treated with the compounds for 3 h. Shoots were isolated and water-stressed. Vertical bars represent SE of the mean. Results of two separate experiments are shown.

kresoxim-methyl or α-NAA in concentrations of $10^{-7}$ to $3 \times 10^{-4}$ M did not alter the *in-vitro* activity of ACC synthase extracted from detached shoots subjected to drought (results not shown). Similarly, the free acid of kresoxim-methyl, which is the first metabolite in wheat, was not active in the enzyme assay. Thus, as previously shown for auxins,[20] kresoxim-methyl could play a regulatory role in the induction process of *de-novo* enzyme synthesis. Contrary to auxins, kresoxim-methyl appears to function only as an inhibiting factor.

### 3.4 Ethylene formation and leaf senescence

Ethylene is involved in responses to stress and in developmental processes such as germination, ripening and senescence.[21] While cytokinins possess senescence-delaying activity,[22,23] ethylene is a well known enhancer of the senescence syndrome, including chlorophyll degradation.[21] Experiments with wheat leaf discs were conducted to show relations between the influence of kresoxim-methyl on ethylene production and leaf senescence. The effects were compared with the

action of AVG, an inhibitor of ACC synthase, the ethylene generator ethephon[21] and the auxins IAA and α-NAA (Figs 7, 8). After exposure of wheat leaf discs to $10^{-5}$ or $10^{-4}$ M kresoxim-methyl for 24 h, ethylene formation was reduced by up to 40% (Fig. 7). Corresponding effects on ethylene formation were induced by $10^{-5}$ M α-NAA and, with less efficiency, IAA. However, at $10^{-4}$ M, the auxins stimulated ethylene production, whereas kresoxim-methyl showed a dose-dependent inhibition which was also observed under dark conditions (not shown). This result corresponds with the effects of the compounds on ACC synthase activity.

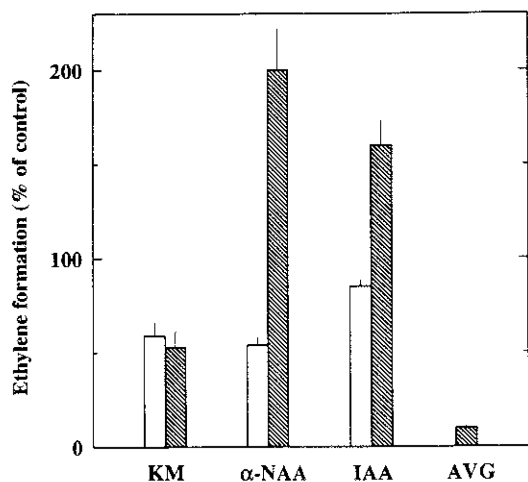


**Fig. 7.** Effects of kresoxim-methyl (KM), 2-(1-naphthyl)acetic acid (α-NAA), indol-3-yl acetic acid (IAA) and aminoethoxyvinylglycine (AVG) on the formation of ethylene in wheat leaf discs after 25 h of treatment. Vertical bars represent SE of the mean.

When leaf discs were incubated in the dark for three days, kresoxim-methyl delayed leaf senescence, characterized by reduced chlorophyll loss, at concentrations of $10^{-4}$ M and above (Fig. 8). α-NAA and IAA were active even at a concentration of $10^{-5}$ M (Fig. 8). AVG, which effectively inhibited ethylene formation (Fig. 7), also retarded leaf senescence, whereas ethephon accelerated this process (Fig. 8). This indicates that the inhibition of ethylene synthesis caused by kresoxim-methyl appears to be related to its senescence-delaying activity.

## 4 CONCLUSIONS

In addition to the fungicidal effect of kresoxim-methyl, physiological and developmental alterations have been observed which could contribute to an improved harvest index in wheat.[5] High yields and a better cereal quality in wheat have also been reported for the strobilurin-type fungicide azoxystrobin.[24] Kresoxim-methyl is thought to increase the area of photosynthetic green tissues and consequently to improve the assimilation rate in wheat by favouring the fixation of carbon dioxide, ultimately through an inhibitory influence on plant respiration (Fig. 9). The latter effect is most likely due to a direct interference of kresoxim-methyl with the mitochondrial electron transport in wheat, because strobilurins have been shown to block the $bc_1$ complex, not only in mitochondrial preparations from yeast, but also from maize.[2] In addition, kresoxim-methyl showed

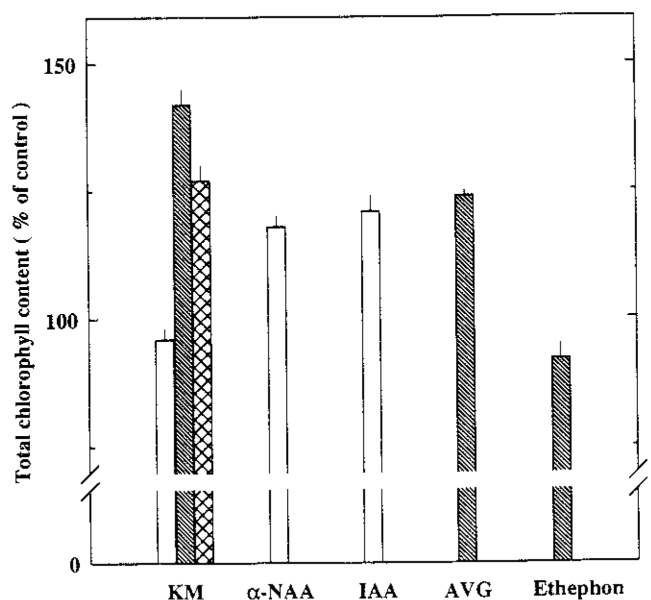


**Fig. 8.** Influence of kresoxim-methyl (KM), indol-3-yl acetic acid (IAA), 2-(1-naphthyl)acetic acid (α-NAA), aminoethoxyvinylglycine (AVG) and ethephon on total chlorophyll content of wheat leaf discs after treatment for three days. Vertical bars represent SE of the mean.

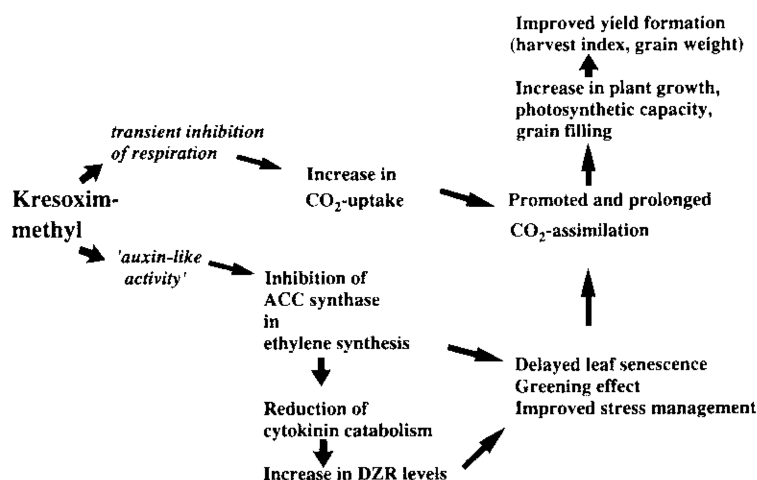


**Fig. 9.** Proposed model of the bioregulatory action of kresoxim-methyl in wheat. The arrows represent a direct or indirect effect on carbon dioxide assimilation and the metabolism of phytohormones and possible relations to growth and yield formation processes. ACC, 1-aminocyclopropane-1-carboxylic acid; DZR, dihydrozeatin riboside.

auxin-like properties in a series of biotests and induced changes in the hormone status in wheat. The most remarkable alteration was the inhibition of ethylene biosynthesis which was demonstrated in leaf discs, intact plants and water-stressed shoots. Kresoxim-methyl affected the rate of conversion of *S*-adenosylmethionine to ACC in the pathway. It is assumed that the compound interfered with the induction process of ACC synthase (Fig. 9), because kresoxim-methyl inhibited effectively the rise in ACC synthase activity in wheat shoots under drought stress, whereas enzyme activity *in vitro* was not influenced by adding the compound. Using four gene-specific probes for ACC synthase[25] (a generous gift of Prof. S. F. Yang, University of Hong Kong), it was shown that kresoxim-methyl did not influence the increase in ACC synthase transcripts in wheat shoots after drought stress (F. Scheltrup, J. Lerchl & K. Grossmann, unpublished results). Therefore, regulation of ACC synthase by kresoxim-methyl might be at the post-transcriptional level. Increased ethylene production is induced in response to a multiplicity of abiotic (e.g. temperature extremes, drought, excessive water, wounding, bending, chemicals) and biotic (e.g. pathogen infection, insect damage) environmental stresses.[21] Ethylene also plays an important role in the regulation of developmental processes, including plant aging and reproductive growth.[21] In crops like wheat, stress ethylene impairs yield, particularly through an acceleration of leaf senescence and the initiation of premature ripening of the grain which cut short the production of assimilates and the duration of grain filling. Consequently, in its function as an inhibitor of ethylene biosynthesis, kresoxim-methyl could alleviate an ethylene-induced over-reaction of the plant to unfavourable environmental stress conditions and thus have a positive influence on yield. The compound could maintain or extend the functioning of the assimilating organs and could prolong and even intensify the partitioning of photosynthetic products to the grain which results in a higher harvest index and improved grain weight (Fig. 9). In the experiments using wheat leaf discs, kresoxim-methyl reduced loss of chlorophyll as a parameter for the progress in senescence. The effect was preceded by an inhibition of ethylene formation. A comparison of the effects elicited by compounds which interfere with ethylene formation (AVG, ethephon) indicated that, in wheat, a reduced rate of ethylene production is, indeed, causally connected with delayed leaf senescence.

Auxins are well known to regulate ethylene production at the level of ACC synthesis. Following the literature, only stimulatory effects of auxins on ethylene biosynthesis through *de-novo* induction of ACC synthase have been reported.[20,21] However, at very low concentrations of auxins such as α-NAA, we observed an inhibition of ACC synthase activity and ethylene formation in wheat. This result correlates with the biphasic behaviour of auxins in the regulation of plant growth: at low concentrations growth is promoted, whereas, at higher levels, phytotoxic inhibition of growth and stimulation of ethylene synthesis are elicited.[18] However, in contrast to auxins, kresoxim-methyl showed only inhibitory activity on ethylene synthesis over a wide range of concentrations. In addition, in treated wheat plants, kresoxim-methyl appeared to shift the hormonal balance in favour of cytokinins rather than of ethylene. Since ethylene has been suggested to accelerate the breakdown of cytokinins[26] through stimulation of cytokinin oxidase activity,[21] reduced levels of ethylene should therefore result in maintained or increased cytokinin contents (Fig. 9). Besides their senescence-delaying potential in wheat leaves,[23] cytokinins are also well-known enhancers of light-induced chlorophyll and thylakoid formation.[27,28] On the other hand, ethylene has been shown to increase chlorophyll catabolism in various leaves.[21] Therefore, the induced

shift in the hormonal balance might also favour chloroplast development and chlorophyll synthesis which could contribute to the intensified green pigmentation (greening effect) of wheat plants exposed to kresoxim-methyl (Fig. 9).

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