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MITOSIS INHIBITION BY A *N*-(1,1-DIMETHYLPROPYNYL) BENZAMIDE SERIES

GÉRARD MERLIN, FRANÇOISE NURIT, PATRICK RAVANEL, JEAN BASTIDE,* CAMILLE COSTE* and MICHEL TISSUT†

Laboratoire de Physiologie cellulaire végétale, Université I de Grenoble, BP 68, 38402 Saint Martin d'Hères cédex, France; *GERAP, Université de Perpignan, Chemin de la Passio Vella, 66025 Perpignan cédex, France

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Key Word Index—Elongation; mitosis inhibition; propyzamide; QSAR; steric hindrance; wheat.

Abstract—A *N*-(1,1-dimethylpropynyl) benzamide series was obtained by changing the substitution of the phenyl on the 3 or 4 position. As is the case for propyzamide, *N*-(1,1-dimethylpropynyl)-3-chlorobenzamide is a powerful and selective inhibitor of mitosis in plant cells at 0.1 μM, as is demonstrated by its characteristic effect on seedlings of five different species (*Triticum sativum* L., *Avena fatua* L., *Lolium multiflorum* Lam., *Raphanus sativus* L., *Sinapis arvensis* L.), by the effects on dividing cells and by the lack of effect on mitochondria or chloroplast activities. Three other compounds of the series inhibit mitosis at 10 μM (3-Br, 3-Me, 3-F). The 4-substituted derivatives, as well as the 3-CN or 3-OMe benzamides have only a small effect on cell division. All these results can be explained by a quantitative structure–activity relationship where the lipophilicity (expressed by π), the electronic activity (expressed by σ) and a steric character expressed by L (illustrating the steric hindrance near the 3 and 4 positions of the phenyl ring) are taken into account. These observations suggest that the 4 position of the phenyl is probably directly involved in the binding of the propyzamide derivatives to a target controlling mitosis in plant cells.

INTRODUCTION

Several different chemicals are known to act on mitosis. Owing to this physiological activity, some of them are now widely used as herbicides (e.g. propham, chlorpropham, propyzamide, trifluraline).

Mitosis can be affected by chemicals in different ways: indirectly, for example, through the disturbance of energy metabolism, or directly by inhibition of the DNA-synthetic phase, i.e. *S* phase (for instance by aphidicolin, [1]) or by *M* phase inhibition. This latter mode of action can be obtained through the effect on several quite different targets: for instance, tubulin self-assembly is inhibited by colchicine [2] and the activity of the microtubule organization centre seems to be changed by propham or chlorpropham [3]. Furthermore, cell wall formation can be inhibited by compounds (such as 2,6-dichlorobenzonitrile [4], coumarin or cellulase [5]) preventing, in some cases, both mitosis and cytokinesis. Moreover, cell division in protoplast or cell suspension cultures is generally controlled by natural or synthetic phytohormones such as 2,4-D and/or cytokinin [6].

Smith *et al.* [7] and Carlson *et al.* [8] have shown that the herbicide propyzamide [*N*-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide] acts in the same way as well-known mitosis inhibitors, in graminaceous seedlings, especially quackgrass. Bartels and Hilton [9] suggested that this compound prevents spindle formation in wheat or corn root cells, therefore interrupting the cell cycle before metaphase. Tissut *et al.* [10] have shown that the powerful effect of propyzamide on mitosis almost disappears when the 4 position of the phenyl ring is substituted by Cl, Br, Me, OMe, NO_2, CN or F. Furthermore, no correlation can be seen between the inhibition of mitosis and the inhibition of the energy-transducing organelles (mitochondria and chloroplasts).

The purpose of the present report is to study the role played by 3-substitution on the phenyl ring of a *N*-(1,1-dimethylpropynyl) benzamide series on mitosis inhibition, and to tentatively suggest a structure–activity relationship.

RESULTS

1. *Effects of* N-(1,1-*dimethylpropynyl*) 3-*chlorobenzamide on the growth of wheat seedlings*

As shown in Fig. 1 wheat seedlings growing in the presence of 10 μM 3-Cl derivative (named 3-Cl benzamide) present a short swollen coleoptile (length: 4 mm) and two or three short swollen roots (total length: 6 mm), 75 hr after treatment. In the untreated seedlings, the length of the coleoptile (and of no. 1 leaf) is 80 ± 5 mm and the total length of the three roots is 28 ± 15 mm.

Microscopic observations of the root apices show numerous dividing cells in the untreated seedlings, with metaphases, anaphases or telophases. On the contrary, in 3-Cl benzamide treated plants, only non-dividing nuclei or dividing nuclei at a prophase-like or post-prophase-like stage are observed (Fig. 1). The quiescent nuclei in this case show an especially large size and are multilobed. All these symptoms are typical of a mitosis inhibition which affects the chromosome migration process, i.e. probably spindle formation or activity.

A further investigation was carried out in order to establish whether such a mitosis disfunction could orig-

†Author to whom correspondence should be addressed.

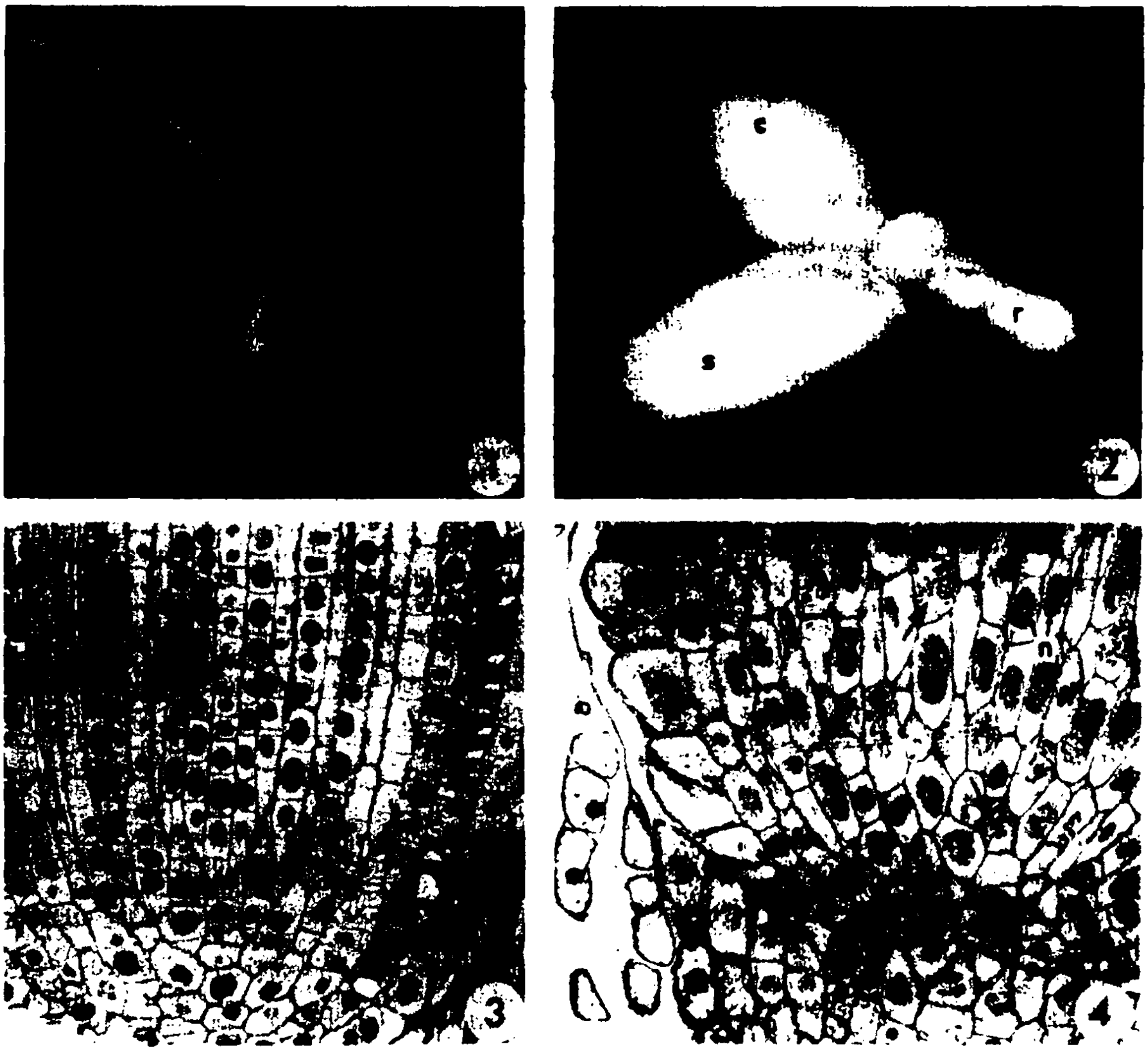


Fig. 1. Effects of the *N*-(1,1-dimethylpropynyl) 3-chlorobenzamide at 10 μM on wheat root cells, as shown by light microscopy. 1. Untreated seedling (× 0.6); 2. Treated seedling (× 5) (c: coleoptile; r: root; s: seed); 3,4. Longitudinal sections of root tips (× 300). 3. Untreated seedling: arrows indicate dividing cells at different stages (metaphase, anaphase, telophase). 4. Treated seedling: arrows indicate abnormal mitosis; in quiescent cells, nuclei (n) are multilobed.

inate from a disturbance in the energy-furnishing processes. For this purpose, potato tuber mitochondria and spinach class A and class C chloroplasts were isolated and their activities studied in the presence of 3-Cl benzamide, following methods previously described [10]. In fact, at 10 μM, 3-Cl benzamide failed to show any effect on these activities. Similar results were obtained with the other derivatives used in this paper [11]. We concluded, therefore, that neither photosynthesis nor mitochondrial respiration were changed by the benzamides studied, up to a concentration of 10 μM. The results obtained on isolated mitochondria reinforce the idea that seedling respiration is unaffected by the benzamides studied, up to a concentration of 10 μM (several of these benzamides being complete inhibitors for mitosis at this concentration).

2. *Effects of the benzamide series on the growth of leaf no.* 1 *and of the roots in wheat seedlings*

Figure 2 shows the effects of the 12 benzamide derivatives studied here, on the coleoptile and no. 1 leaf elongation in etiolated wheat seedlings. All of the efficient derivatives (3,5-di-Cl, 3-Br, 3-Me, 3-F) induce the same symptoms as described for the 3-Cl benzamide, thus suggesting the same mode of action, i.e. selective mitosis inhibition. In the case of derivatives with low efficiency, such as 3-NO_2 or 4-OMe benzamide, the symptoms become more obvious (no clear coleoptile swelling), suggesting that a possible mitosis inhibition, obtained at high concentration, may be accompanied by side effects on other physiological activities.

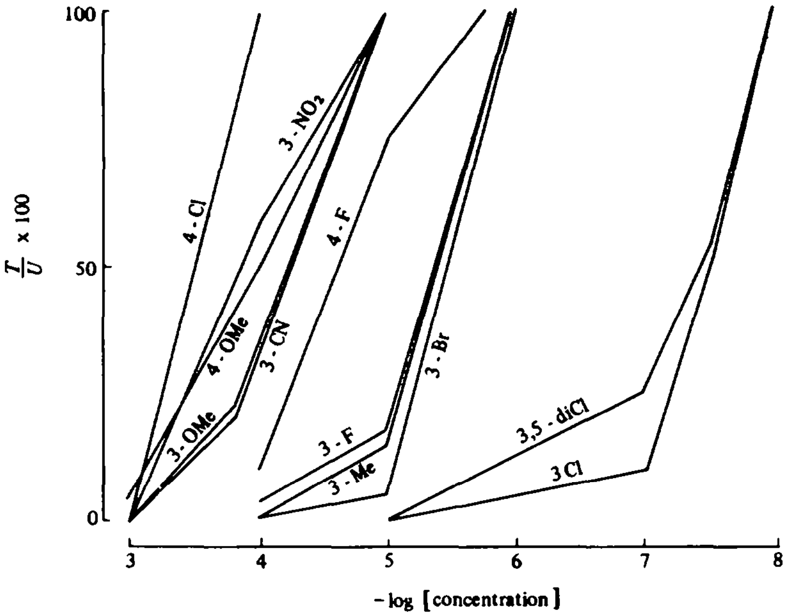


Fig. 2. Effect of the benzamide derivatives at different concentrations on the wheat no. 1 leaf length, 75 hr after treatment. The length of the leaf of treated samples (T) is expressed as a percentage of the length of untreated seedlings (U). Each point represents the mean value of 10 measurements. The experiment was done in triplicate.

Among the 12 studied derivatives, their efficiency as mitosis inhibitors presents at least a 10000-fold change, depending on phenyl substitution. As mentioned before [10], substitution at the 4 position of the phenyl considerably lowers the inhibitory efficiency. In contrast, 3-substituted derivatives have, in most cases, a potent inhibitory activity on mitosis. However, the nature of the substituent on the 3 position is of great importance: for example, a 120-fold greater effect is obtained when a Cl replaces Br, Me or F. Moreover, 3-substitution by CN, OMe or NO_2 gives almost ineffective derivatives. 3,5-Disubstitution by Cl (propyzamide) leads to a product, the efficiency of which is the same as that of the 3-Cl derivative (Table 1).

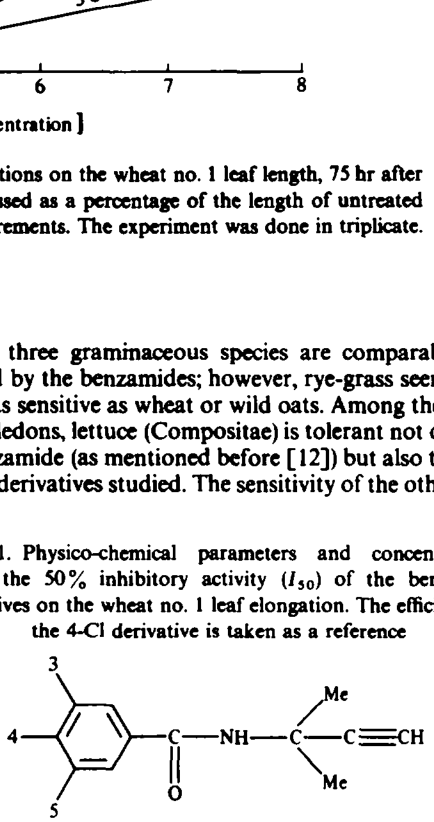
Table 2 shows the effects of the benzamides, at 100 μM, on no. 1 leaf elongation, root elongation and the dry weight (seed excluded) of the seedlings. The inhibition of elongation affects the coleoptile, the no. 1 leaf and the roots at the same time. The increase in dry weight of the growing parts of the seedlings is affected to a much lesser extent than the elongation.

3. *Effects of the benzamide series on growth of seedlings of several plant species*

Table 3 summarizes the effects of the derivatives studied at a concentration of 100 μM, on the growth of seedlings of three monocotyledons (wheat, wild oats, rye-grass), and three dicotyledons (radish, mustard, lettuce). Three classes have been defined, according to the sensitivity of each plant species to each benzamide derivative: derivatives with a full inhibitory effect at 100 μM are in class I; class II derivatives induce only a partial inhibition at 100 μM; class III derivatives are without effect at 100 μM.

The three graminaceous species are comparably inhibited by the benzamides; however, rye-grass seems not to be as sensitive as wheat or wild oats. Among the three dicotyledons, lettuce (Compositae) is tolerant not only to propyzamide (as mentioned before [12]) but also to each of the derivatives studied. The sensitivity of the other two

Table 1. Physico-chemical parameters and concentrations giving the 50% inhibitory activity (I_{50}) of the benzamide derivatives on the wheat no. 1 leaf elongation. The efficiency of the 4-Cl derivative is taken as a reference



	L	π	σ	I_{50} (μM)	Efficiency
4-NO_2	—	—	—	> 1000	—
4-Cl	3.52	0.71	0.23	500	1
4-OMe	3.98	− 0.02	− 0.27	200	2.5
3-NO_2	—	—	—	100	5
3-CN	2.98	− 0.57	0.56	60	8.5
3-OMe	2.04	− 0.02	0.12	60	8.5
4-F	2.65	0.14	0.06	40	12.5
3-Br	2.19	0.86	0.39	6	85
3-Me	2.15	0.56	− 0.07	6	85
3-F	1.30	0.14	0.34	6	85
3,5-di Cl	1.96	1.42	0.74	0.05	10.000
3-Cl	1.96	0.71	0.37	0.05	10.000

Table 2. Effects of the benzamide series at 100 μM on no. 1 leaf and root elongation, and on dry weight of the wheat seedlings, 75 hr after treatment

Benzamide derivatives	Leaf no. 1 length	Roots length	Dry weight (seed excluded)
Reference	100	100	100
4-NO_2	100	25	65
4-Cl	100	72	100
4-OMe	44	30	55
3-NO_2	44	25	70
3-CN	6	3	32
3-OMe	15	4	46
4-F	12	6	42
3-Br	4	2	50
3-Me	6	8	43
3-F	6	4	38
3,5-di Cl	4	2	55
3-Cl	5	2	33

Table 3. Effects of the benzamide series at 100 μM on the seedlings of six different species

	Class I	Class II	Class III
4-NO_2		6	1, 5
4-Cl			1, 3, 4, 5, 6
4-OMe		1	3, 4, 6
3-NO_2		1, 2	3, 4, 6
3-CN		1, 2	3, 4, 5, 6
3-OMe		1, 2, 4	3, 6
4-F		1, 2	5, 6
3-Br	1, 2, 3, 4, 5		6
3-Me	1, 2, 3, 5		4, 6
3-F	1, 2, 3, 5	4	6
3, 5-di Cl	1, 2, 3, 4, 5	6	
3-Cl	1, 2, 3, 4, 5		6

The six different species are represented by numbers from 1 to 6. 1: wheat (*Triticum sativum* L.), 2: wild oats (*Avena fatua* L.), 3: rye-grass (*Lolium multiflorum* Lam.), 4: radish (*Raphanus sativus* L.), 5: mustard (*Sinapis arvensis* L.), 6: lettuce (*Lactuca sativa* L.).

Class I: full inhibitory effect at 100 μM; Class II: partial inhibitory effect at 100 μM; Class III: no effect at 100 μM.

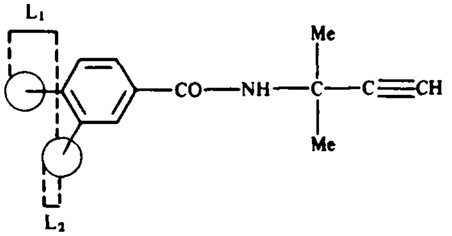


Fig. 3. Scheme illustrating the mode of determination of the *L* parameter.

species (mustard and radish: Cruciferae) is near that of wheat, wild oats or rye-grass.

4. *Relation between structural parameters and efficiency on wheat no. 1 leaf elongation*

In the series studied, a quantitative relationship (QSAR) between the inhibitory activity on wheat leaf elongation and different structural parameters was looked for. For this purpose, several physico-chemical parameters were measured: π is a parameter giving a quantitative measurement of the lipophilicity of the molecule [13]. It represents the partition coefficient between octanol and water in standard conditions. σ is an electronic parameter [14]. Its value was obtained by using the hydrolysis constant of the benzamide in alkaline media [15]. L is a parameter of the same nature as the sterimol parameters of Verloop and Tipker [16]. It is illustrated by Fig. 3. Taft's E_s was measured as described by Mager *et al.* [17].

Table 1 indicates the values of the parameters π, σ and L. A QSAR study taking into account these three parameters leads to the following best equation: $\log 1/I_{50} = 5.137 - 0.569\, L + 1.192\, \pi + 1.253\, \sigma$: $n = 10$; $r = 0.77$; $F = 5.18$ (where I_{50} is the concentration giving 50% inhibition of wheat no. 1 leaf elongation, n is the number of compounds studied, r is the correlation coefficient and F the statistic test indicating the significance of the correlation found). No satisfactory result was obtained either with π and σ alone or after addition of the Taft's steric parameter E_s [17]. In the equation, NO_2 derivatives were excluded, as was the case for other QSAR studies [15] because of significant secondary effects on plant metabolism.

DISCUSSION

The benzamides studied here contain characteristic mitosis inhibitors. There substances (e.g. propyzamide or 3-Cl benzamide) have no effect on the activities of isolated chloroplasts and mitochondria, at concentrations which fully inhibit mitosis. The inhibition of cell division is certainly not, therefore, an indirect effect induced by a perturbation of the energy transducing processes. As mitosis is inhibited between chromosome formation and metaphase, spindle formation or activity could be a possible target. In the 4- or 3-substituted benzamides studied here, the inhibitory activity on mitosis changes greatly; the comparison between 3-Cl, 4-Cl and 3,5-di-Cl derivatives illustrates the structural requirement for a potent effect on cell division: for 3-Cl and 4-Cl derivatives, the values of π, on the one hand, and σ, on the other hand, are similar or comparable. The great difference in efficiency between these two compounds is associated with a great change in the L value, demonstrating that the steric hindrance in the 3 and the 4 positions is directly involved in controlling their effect on mitosis. Moreover, the 3,5-di-Cl derivative which presents a π and σ much higher than the mono-chlorinated compounds, but the same value of L as the 3-Cl compound, shows the same effect as this last derivative on mitosis.

Replacing the Cl by other substituents on the 3 position of the phenyl leads to a more complex situation: with CN substitution, the values of L and π together explain the lower efficiency; when OMe is the substituent, the value of L appears to be near to that of the 3-Cl derivative, but a low lipophilicity shown by the low value of π probably hinders the movement of this derivative through the biological membranes, and therefore lowers its efficiency. Another explanation may be that the target possesses a hydrophobic part which could be more repulsive for the derivatives substituted by OMe than by Cl or Me.

In the case of the fluorinated derivatives, substitution by F on the 4-position, as compared to that on the 3-position, gives a value of L which is too high for a good efficiency, but also a 0.3 decrease in σ, which is acting in the same way. Furthermore, the two types of F-derivatives have a low lipophilicity ($\pi = 0.14$) which may alone explain a 120-fold jump in efficiency from the 3-F benzamide to the 3-Cl derivative.

The equation obtained with the parameters L, π and σ, although not of the best quality ($r = 0.77$), agrees with a 10 000-fold change in efficiency in the series studied, when the phenyl substitution changes. Moreover, it reinforces the demonstration of the role played by the L parameter in mitosis inhibition, and strongly suggests that the free 4-position of the phenyl is one of the binding points of the benzamide to its site of action, which controls mitosis. It is interesting to note that a similar structural requirement, in order to obtain mitosis inhibition, has also been found in a carbamate series [18]. The mode of action of a well-known member of this series, the isopropyl N-(3-chlorophenyl) carbamate (CIPC), has been extensively studied. It seems to act in some manner on the microtubule organization centres of the spindle, perhaps disturbing their microtubule organizing capacity and/or duplication [18, 19]. When we consider the compared efficiency between carbamates and benzamides as being dependent on the 3- and 4-substitution of the phenyl ring, the analogy found in our work, between the two series, strongly suggests a similar mode of action.

One part of our results agrees with those obtained by Swithenbank *et al.* [20] through routine greenhouse testing, carried out for 14 days on 20 different plant species. 3-Cl was better than 4-Cl substitution in their test, and the 3,5-$(OMe)_2$ derivative was fully ineffective. However, their test which was (1) carried out for a long time, (2) after soil treatment and (3) only evaluating a total herbicidal effect, cannot be further compared with our experiment.

EXPERIMENTAL

Culture of seedlings. Seeds of wheat (*Triticum sativum* L., var. Darius), wild oats (*Avena fatua* L.), rye-grass (*Lolium multiflorum* Lam), radish (*Raphanus sativus* L., var. Rose à bout blanc), mustard (*Sinapis arvensis* L.) and lettuce (*Lactuca sativa* L., var. Grosse blonde paresseuse) were rinsed with sodium hypochlorite and placed on filter paper in sterilized Petri dishes. The benzamides dissolved in dimethylsulphoxide were added in H_2O and the cultures were incubated for 5 days, at 28° in the dark.

Mitochondria and chloroplast isolation. Potato tuber mitochondria and spinach leaf chloroplasts were isolated as described in ref. [10].

QSAR calculations. The classical empirical equation of ref. [21], which describes relationships between biological activity and chemical structure, can be written as follows: $\log 1/C$: $k_1X + k_2X^2 + k_3Y + k_4Z + k_5$; where C is the molar concentration that provides a given biological activity (here I_{50}), X is a parameter that expresses hydrophobicity, Y is a parameter that expresses electronic effects, Z is a parameter that expresses steric effects and k_1, k_2, k_3 etc are the regression coefficients obtained by fitting the equation to experimental data. For this purpose, a stepwise multiple linear regressive analysis was performed on a 9825 A Hewlett-Packard computer as described previously [22].

Chemicals. The synthesis of the substituted benzamides was performed as described in ref. [15]. The different derivatives were crystallized several times and their purity controlled by TLC and HPLC. The 0.1 M solutions of benzamides in DMSO were diluted in the reaction media. DMSO never exceeded 1% in our biological tests. It had a limited effect on the seedling growth at this concentration. At 0.1%, it was without effect on this test. The presence of large amounts of intact products in the culture medium for seedlings was demonstrated by TLC at the end of the experiments.

Microscopic observations. Wheat roots were fixed and embedded according to a technique used for electron microscopy and described in ref. [23]. Sections, 1 μM in thickness, were stained according to ref. [24].

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