Pergamon

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CHEMICAL BASIS OF THE RESISTANCE OF BARLEY SEEDS TO PATHOGENIC FUNGI

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Key Word Index—*Hordeum vulgare*; Gramineae; seeds; epicuticular wax; alkylresorcinols; pathogenic fungi; resistance.

Abstract—The 5-(*n*)-alkylresorcinol fraction of the epicuticular waxes of *Hordeum vulgare* seeds appeared to be responsible for their in-born resistance to pathogenic fungi such as *Aspergillus niger* and *Penicillium crysogenum*. The antifungal properties of this fraction were evaluated qualitatively and quantitatively with a novel bioassay where the extreme lipophilicity of these compounds was taken into account. The minimum inhibitory concentration in the fungi tested ranged from 5.6 to 10 μg cm^{-2} for the alkyresorcinols. The behaviour of the different cultivars against these fungi could be predicted by measuring the natural amount of resorcinols of each variety by TLC-scanning densitometry. The ranking of cultivars thus established correlated well with the field behaviour of each cultivar, providing a useful and rapid method for predicting the behaviour against fungi of new varieties being developed. Copyright © 1997 Elsevier Science Ltd

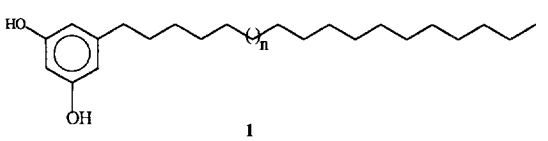
INTRODUCTION

The interphase of a plant with its ecosystem is established at the waxy epicuticular layer of leaves, the bark and the surface of roots. Chemicals found in these organs are biosynthesized specifically to protect the plant against external aggression and predators. Of these chemicals, those with antifungal and antibacterial properties are important because fungal spores have to germinate on the plant surface prior to their invasion [1]. In the case of leaves, antifungal compounds have been found in epicuticular waxes from different species and their activity against different fungi has been evaluated [2–4]. This is of particular interest in the case of seeds of the Poaceae, because in a natural environment they must survive on the ground in different moisture conditions and in a non-sterile milieu, until germination can occur [5].

Cereal seeds contain varying amounts of 5-(*n*)-alkylresorcinols (**1**), which are located in the epicuticular layer of the seed shell and the kernel itself [6–9]. The resorcinol structure allows the prediction of their antifungal activity. They have been associated with the resistance of unripe mango fruit against fungi [10]. They have also been isolated from *Streptomyces cyaneus* (adipostatins A and B). These compounds are potent inhibitors of glycerol-3-phosphate dehydrogenase [11].

The seeds, such as those of wheat, maize, barley and other cereals, are of primary importance in human food and animal feeds. The grains have to be stored without treatment with commercial fungicides, as these products can be dangerous to public health. In the case of barley, malting can be affected by added chemicals. Conversely, the sanitary condition of the seeds is the key to healthy crops and good harvest yields. The selection of less susceptible cultivars with in-born resistance to fungi can therefore be of great importance in assuring better microbiological quality of the final foodstuffs, while minimizing the use of agrochemicals.

In this report, we examine the correlation between the amount of 5-(*n*)-alkylresorcinols in epicuticular waxes of seeds of *Hordeum vulgare*, with their resistance to the common fungi, *Aspergillus niger* and *Penicillium crysogenum*.



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RESULTS AND DISCUSSION

Seven different cultivars of barley commonly planted in Uruguay [12] were analysed for their content of alkylresorcinols using TLC-densitometry of the spots after colour development with Fast Blue B. In order to compare levels of these compounds with the results of the bioassays, the total area of the seeds of each cultivar

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Table 1. Content of 5-(*n*)-alkylresorcinols in different varieties of barley planted in Uruguay

Cultivar	Seed (% of weight)	Area of seed (cm^2)	Concn alkyl resorcinols (mg cm^{-2})	Observed behaviour in silos*
Stirling	0.024	0.61	6.10	g
Clipper	0.043	0.71	3.85	a
FNCI	0.033	0.67	3.21	r
Bowmann	0.048	0.66	16.05	vg
CLE	0.027	0.67	4.82	a–g
MN599	0.026	0.67	9.63	g
Ana	0.029	0.71	8.99	a–r

*r = regular (3% < is < 5%); a = acceptable (2% < is < 3%); g = good (1% < is < 2%); vg = very good (is < 1%); is = infected seeds. Criteria of evaluation: visual inspection of sample [12; Capetini, personal communication].

was also evaluated (see Experimental). The amount of resorcinols per seed was then expressed as resorcinols cm^{-2}. The values in Table 1 represent the average of three determinations with three repetitions for each; variation was always below 1% for each determination. From the cultivars studied, cv. Bowmann had the highest content of alkylresorcinols in the epicuticular wax of the complete seed, followed by cv. MN599 and cv. Ana (Table 1).

The antifungal activity of natural alkylresorcinols could be confirmed using the TLC bioassay described by Hargreaves *et al.* [4], with some modifications. Air oxidation of the alkylresorcinols on silica gel plates was avoided by imbibition of the plate by a previous development with a solution of 5% Petrolatum in petrol. The Petrolatum covering the adsorbent simulates the lipid environment of the seed's epicuticular wax. After development, the plate was treated with an inoculum of 10^{10} spores ml^{-1} and incubated in a moist chamber at 27°. After three days, the most visible inhibition of fungal growth was at the resorcinol spot. This bioassay was also useful to evaluate minimum inhibitory concentration (MICs). Careful selection of the chromatographic conditions (see Experimental) resulted in very regular round spots for the resorcinols, so that the areas could be measured as circular (spots were observed under UV light). Completing the bioassay with increasing amounts of alkylresorcinols from *H. vulgare* applied to the plate, a MIC in $\mu g\ cm^{-2}$ could be established (Table 2). As the layer of silica gel is 0.25 mm thick, the real content of alkylresorcinols applied cannot be properly evaluated, as an important proportion is not on the surface.

A new bioassay was then developed in order to evaluate the activity of the compounds, which were placed as a film over the surface. Each determination was carried in five small tubes of 1.0 cm internal diameter. One contained a positive inhibition blank, another was a positive growth blank and the other three were repetitions of a given concentration of the tested compound. Alkylresorcinols were placed to cover the bottom and were covered with a film of Petrolatum to avoid oxidation.

Alkylresorcinols inhibited the growth of *A. niger* at 5.6 $\mu g\ cm^{-2}$ and that of *P. crysogenum* at 10 $\mu g\ cm^{-2}$ using the small-tube test (Table 2). These figures were of the same order but lower than those obtained with the TLC test. The reason for this deviation is the fact that the alkylresorcinols applied to TLC plates form a 'cylinder' 25 mm high and only the activity at the surface is being measured. Comparison between both MICs is shown in Fig. 1. A value of 1 implies that the amount of alkylresorcinols per unit area is the same as the required MIC. Barley cultivars were ranked for their content of alkylresorcinols in seed waxes (Table 1) and compared with the fungal damage observed in silos.

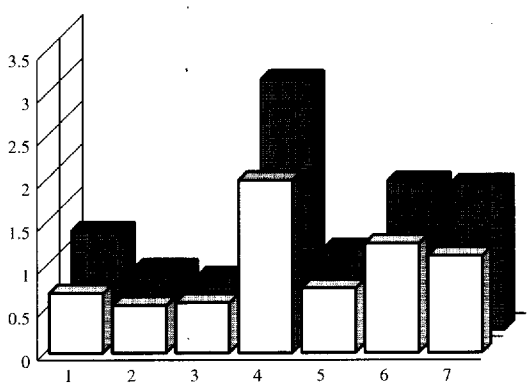

Fig. 1. MICs for seeds of seven barley cultivars grown in Uruguay. Cultivar: 1, Stirling; 2, Clipper; 3, FNCI; 4, Bowmann; 5, CLE 116; 6, MN599; 7, Ana; white, TLC; black, STT.

Table 2. MIC for the fungi tested using both bioassays

Fungi	TLC-MIC mg cm^{-2}	STT-MIC mg cm^{-2}
Aspergillus niger	8.5	5.6
Penicillium crysogenum	15.3	11.0

This criterion showed a good correlation. Bowmann was the cultivar that had the best sanitary quality with cvs Stirling and MN599 showing intermediate behaviour. The cv. Ana was more complex because although there was a high content of alkylresorcinols in the seed, this cultivar proved to be very sensitive to fungal attack at the plant stage [12, 13, Flano Capetini, personal communication]. Our technique provides a simple and reliable method for the prediction of field performance of new cultivars in breeding programmes leading to the selection of seeds resistant to fungal attack.

EXPERIMENTAL

General. Reagent quality solvents were redistilled from glass prior to use. TLC plates and flash CC quality silica gel were from Macherey–Nagel. TLC scans were performed using a flying spot densitometer in the refractance mode at 545 nm. UV spectra were determined on a Shimadzu UV-210 dual wavelength spectrophotometer. All the calculations were done assuming a normal distribution, checked by Barlett's variance test.

Isolation of wax. Seeds (100 g) were dipped in $CHCl_3$ for 30 sec. Solvent was evapd under red. pres. and the sealed wax extract was kept at −20° until use.

Quantification of alkylresorcinols. On a 20 × 20 TLC plate, 2, 4, 8, 10 and 12 μg of *H. vulgare* alkylresorcinols (calibration curve) and 10 μg (replicated three times) of the test wax were spotted. The plate was bidimensionally–unidirectionally developed using CH_2Cl_2–MeOH (97:3) for the first 10 cm and then toluene–petrol (1:4) to 18 cm.

After spraying with Fast Blue B, the area of the red spots corresponding to the alkylresorcinols was quantified at $\lambda = 545$ nm with the densitometer. The amount of alkyl resorcinols was evaluated using a linear regression only when the correlation coefficent was > 0.9993. The reported values are the average of three separate runs.

The seed weights of the different cultivars were obtained by weighing five samples of 100 seeds each and averaging the results. To determine the average surface area of the seeds of the different barley cvs, individual seeds of three samples of 20 seeds of each cultivar were measured along their longitudinal and transversal axes. Considering the seeds as revolving ellipsoids, the area of seed surface was calculated using the formula $S = 2\pi b^2 + (2\pi ab/\epsilon)$ arcsin ϵ, where $\epsilon = \sqrt{a^2 - b^2}/a$ and $a > b$. By assuming an even distribution of the resorcinols on all the surface of the seeds, the values in Table 1 were obtained by dividing the content of resorcinols per seed per calculated area.

Isolation of alkylresorcinols. Seed wax (100 mg) was chromatographed on a silica gel column using CH_2Cl_2 with an increasing percentage of MeOH as mobile phase. Frs corresponding to alkylresorcinols were collected and their homogeneity checked by TLC, IR and UV. The resorcinols thus obtained were used in bioassays.

Bioassays. Aspergillus niger and *P. crysogenum* were isolated from *H. vulgare* seeds and kept on malt agar.

(a) *TLC Method* (1). Determination of active frs: 5 μl of a 1 mg/ml^{-1} CH_2Cl_2 soln of seed wax were spotted on a TLC plate in triplicate. The plate was developed as described under quantification. After drying, the plate was imbibed by a second full run with a 5% soln of Petrolatum in petrol. The plate was immersed in 20 ml of a soln containing 10^{10} spores ml^{-1} for each fungus on minimal medium BG-11 with 5% glucose, and incubated for 48 hr at 27° in a moist saturated chamber. Inhibition zones were seen as white spots at the R_f value corresponding with alkylresorcinols. (2) MIC determination: 1–10 mg of alkylresorcinols from *H. vulgare* were spotted onto a 20 × 20 cm TLC plate, chromatographed and incubated as described above. Resorcinol spots were visualized under UV light. Using this solvent system, each spot had round regular shape and the area of the spot was measured as a simple circle, $a = \pi r^2$. MIC was taken as the minimal concentration per area of spot of alkylresorcinols that inhibited fungal growth on the plate.

(b) *Small-tube test.* Five flat bottom tubes 1 cm high × 1 cm i.d. were sterilized. In three of them, the desired amount of alkylresorcinols was placed in CH_2Cl_2 solution (substance to be tested), in the fourth tube 5 μl of a 5% solution of *o*-phenyl phenol (positive inhibition blank) and in the fifth tube, 5 ml of CH_2Cl_2 (positive growth blank). Solvent was then allowed to evaporate in a laminar flow chamber; 5 μl of a 5 % soln of Petrolatum in petrol were then introduced into the five tubes and the solvent allowed to evaporate. Tubes were then inoculated with 1 ml of a suspension containing 10^6 spores; ml^{-1} and incubated at 27° for 48 hr. Growth and inhibition were checked by inspection under the light microscope.

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