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TOXICITY OF 6-METHOXYMELLEIN AND 6-HYDROXYMELLEIN TO THE PRODUCING CARROT CELLS

FLAVIA MARINELLI,* UGO ZANELLI† and VITTORIA NUTI RONCHI†

Scuola Normale Superiore, P. zza Cavalieri 7, 56100 Pisa, Italy; †Istituto di Mutagenesi e Differenziamento, CNR, Via Svezia 10, 56124 Pisa, Italy

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Key Word Index—*Daucus carota*; Umbelliferae; suspension cultures; phytoalexin; 6-methoxymellein; 6-hydroxymellein; toxicity.

Abstract—6-Methoxymellein (6-MM) is the carrot phytoalexin and 6-hydroxymellein (6-HM) its immediate biosynthetic precursor. 6-MM is more toxic than 6-HM to the producing carrot cells, as demonstrated by the differential toxicity of these two dihydroisocoumarins to either colony forming units in solid medium or cell viability and mitotic activity in suspension cultures.

INTRODUCTION

Phytoalexins are low-molecular-mass compounds produced by plant tissues in response to microbial invasion or after treatment with various biotic and abiotic elicitors [1, 2]. These compounds prevent the growth of most fungi and some bacteria, but they are also toxic to the producing plant cells [2–7].

Carrot phytoalexin, a dihydroisocoumarin known as 6-methoxymellein (6-MM) is accumulated in carrot roots infected by various fungi and in cell suspension cultures treated with pronase, pectinolytic enzymes, oligosaccharides from carrot cell walls and several chemical agents [4, 8–12]. In previous papers [11, 12], we described how the structurally related dihydroisocoumarin 6-hydroxymellein (6-HM) is also produced by elicitor-treated carrot cells. For this compound a double role was proposed either as the immediate biosynthetic precursor of 6-MM [13, 14], or one of the products of the 6-MM detoxication metabolism by carrot cells themselves [4].

The present study was undertaken to investigate differences in the toxicity of 6-HM and 6-MM to the producing carrot cells, with the aim of understanding the physiological and metabolic correlation between these two dihydroisocoumarins in the carrot response to elicitation.

RESULTS

As shown in Fig. 1, the dose-dependent effect of 6-MM and 6-HM to carrot cells was measured as reduction of colony forming units (C.F.U.) in solid medium. ED_{50}‡ values for 6-MM and 6-HM were 0.032 and 0.07 mM, respectively.

Cytotoxicity of the two dihydroisocoumarins was investigated by adding different concentrations of 6-MM and 6-HM to growing carrot cells in liquid medium. As shown in Fig. 2A, the addition of 6-MM at 0.06, 0.12 and 0.3 mM reduced carrot cell viability to 30–40% after five days of treatment. After 15 days incubated with 0.12 and 0.3 mM respectively, only 2% and 8% of cells survived, whereas with 0.06 mM about 60% were viable. The addition of 6-HM at 0.06, 0.12 and 0.3 mM reduced cell viability to 40–50% after five days of treatment, but after 15 days a rapid recovery of 70–90% was observed (Fig. 2B).

Differential cytotoxicity of the two dihydroisocoumarins was confirmed by studying their effect on mitotic activity. As reported in Table 1, 0.12 and 0.3 mM of 6-MM blocked mitotic activity. At a concentration of 0.06 mM the effect of 6-MM was less drastic and a partial recovery was observed after the ninth day of treatment. The addition of 6-HM reduced the mitotic index during the first week of incubation, but after the ninth day no difference was observed between the treated and the control cells.

DISCUSSION

The results described in this paper show that 6-MM is more toxic than 6-HM to carrot cells. The range of effective concentrations for both the dihydroiso-

*Correspondence at the present address: Flavia Marinelli, Lepetit Research Center, Via R. Lepetit 34, 21040 Gerenzano (Varese), Italy.

‡ED_{50} is the dose of the assayed compound which reduces the number of C.F.U. to 50%.

Table 1. Effect of 6-MM and 6-HM on the mitotic index (no. cells in mitosis/no. cells counted) of suspension-cultured carrot cells after the Feulgen reaction

Phytoalexin (mM)	Days of treatment 2nd	5th	7th	9th
Control	2.5	1.5	2.8	2.4
0.06 6-MM	2.7	0.9	0	1.9
0.12 6-MM	0.5	0	0	0
0.3 6-MM	0	0	0	0
0.06 6-HM	1.7	1.7	1.6	2.7
0.12 6-HM	1.2	1.4	1.7	2.4
0.3 6-HM	1.2	1.0	0.7	2.9

Values represent the mean of three measurements on samples obtained from three replicate experiments. At least 1000 cells were analysed in each measurement.

coumarins falls in the order of magnitude of 0.01 to 0.1 mM, in which various phytoalexins are toxic to microorganisms and plant cells [1, 2]. 6-MM inhibits the growth of fungi and bacteria at concentrations ranging from 0.05 to 0.5 mM [8, 15]. Its ED_{50} values for the inhibition of mycelium growth and spore germination of the carrot pathogen *Botrytis cinerea* are 0.7 mM and 0.5 mM, respectively [16, 17]. Kurosaki *et al.* reported that 6-MM inhibits growth of carrot cells at concentrations above 0.1 mM, causing a rapid and irreversible decrease in viable cell number at about 0.5 mM [4]. To our knowledge, no data were previously reported on the toxicity of 6-HM to either microorganisms or plant cells. Coxon *et al.* [18] and Superchi *et al.* [19] described a differential effect of the two dihydroisocoumarins to *Artemia salina* and Chinese hamster *in vitro* cells. 6-MM and 6-HM ED_{50} values

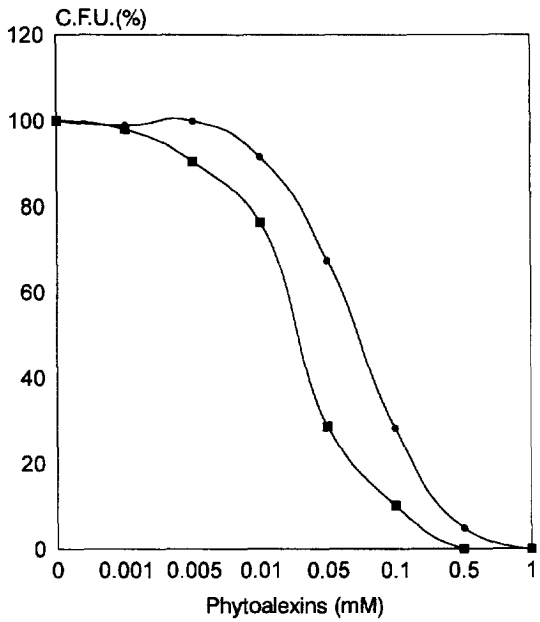

Fig. 1. Toxic effect of 6-MM (■) and 6-HM (●) measured as reduction of carrot C.F.U. in solid medium. Values were calculated as the mean of ten replicates for each concentration and as a percentage of the number of control colonies.

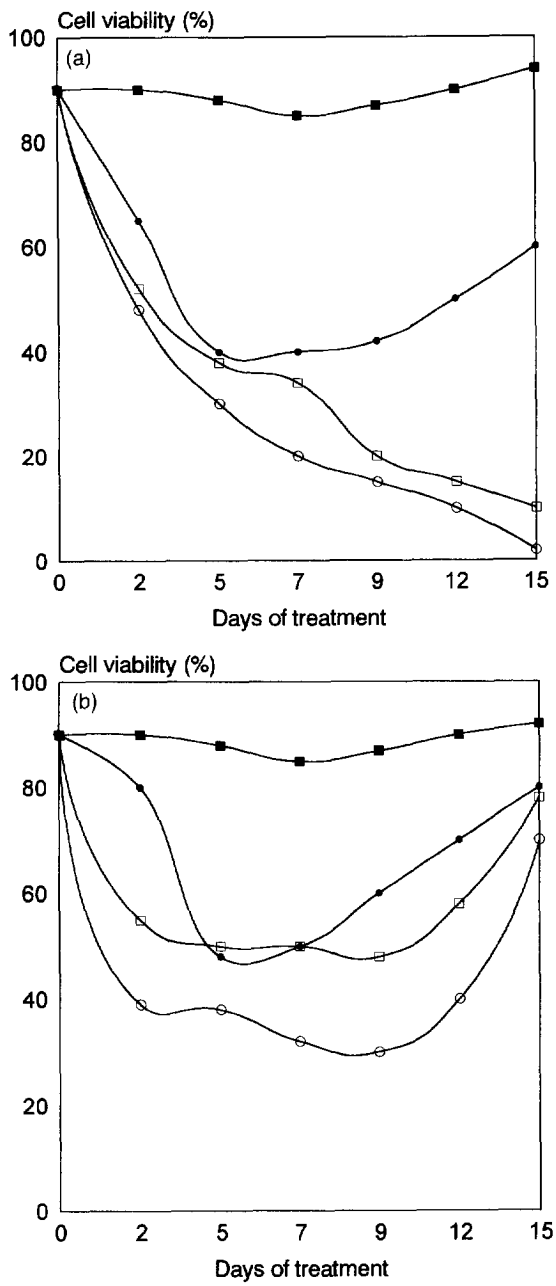

Fig. 2. Effect of 6-MM (a) and 6-HM (b) to viability of suspension-cultured carrot cells, detected by fluorescein diacetate vital stain. The symbols represent 0 (■), 0.06 (●), 0.12 (□) and 0.3 (○) mM 6-MM or 6-HM. Values were calculated as the mean of three measurements on the samples obtained from three replicate experiments. At least 1000 cells were analysed in each measurement.

were 0.43 and 0.66 mM for *Artemia salina* and 0.46 and 0.66 mM for Chinese hamster cells.

These data which compare the effect of 6-MM and 6-HM on various cells/organisms and show certain variability due to the different experimental systems, indicate that the *O*-methylation of phenol group at 6-position confers a higher toxicity to the dihydroisocoumarin molecule. The difference may be due to an

increased liposolubility of 6-MM, resulting in an improved mobility across cellular membranes. This would be in agreement with the hypothesis that the biochemical basis of phytoalexin toxicity is due to the alteration of cell membrane integrity [1, 2, 4, 20].

We previously reported that 6-HM is produced inside the elicitor-treated carrot cells, whereas 6-MM is accumulated in the culture medium [11, 12]. *O*-methylation of 6-HM is described as the rate-limiting step in the carrot phytoalexin biosynthesis whereas the demethylation of 6-MM has been suggested as a way of reducing phytoalexin self-toxicity [13]. The differential toxicity of the two dihydroisocoumarins suggests the idea that carrot cells can modulate the production of 6-MM in response to elicitation by controlling its biosynthesis from and its degradation to the less toxic 6-HM.

EXPERIMENTAL

Carrot cell culture. Cell suspension cultures of *Daucus carota* cv. S. Valery were obtained and maintained in Gamborg's B5 medium as previously described [11, 12].

Preparation of 6-MM and 6-HM. 6-MM and 6-HM were isolated and purified from carrot roots infected by the fungus *Fusarium solani* as previously described [11]. Larger amounts of 6-HM were prepared by demethylating 6-MM with BBr_3 in dry CH_2Cl_2 [4, 11]. Identity and purity of 6-MM and 6-HM were checked by HPLC, UV, CD, ^{1}H-NMR and EIMS [11].

Toxicity of phytoalexins to carrot cells in solid medium. 50 ml of four-day-old carrot cell suspension was filtered through a 120 μm filter. Single cells and small clumps were collected, counted in a Nageotte camera and inoculated (10^2/ml) in Gamborg's B5 medium containing 0.8% (w/v) agar and different amounts of 6-MM and 6-HM dissolved in DMSO. DMSO at the same final conc. (0.1%) was added to control cultures. Colonies were counted after 20 days of incubation at 25°C.

Toxicity of phytoalexins to carrot cells in liquid medium. 50 ml of 4-day-old carrot cell suspension was filtered and counted as above described. Single cells and small clumps were inoculated (2×10^4) in 100 ml Gamborg's B5 medium containing different amounts of 6-MM and 6-HM dissolved in DMSO. DMSO at the same final concn (0.1%) was added to control cultures. Flasks were incubated at 25°C on a rotatory shaker at 80 rpm and 5 ml culture samples were collected at different times of growth. The mitotic index (n° cells in mitosis/n° cells counted) was calculated by counting nuclei in different phases of the cell cycle after the Feulgen reaction [21]. Cell viability was measured by counting the fluorescent and non-fluorescent cells after incubation with the fluorescein diacetate vital stain according to the method previously described [12, 22].

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