

Ascorbic acid induces furanocoumarin production in organ cultures of *Glehnia littoralis*

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Abstract

Exogenously supplied ascorbic acid (AsA) strongly induced furanocoumarin production in leaf and root cultures of *Glehnia littoralis*, but not in cell suspension cultures, after 24 h of treatment. The dose dependency showed that both organ tissues responded well to AsA supplied at concentrations of 10-40 mM. For induction of furanocoumarin production, roots required contact with AsA for at least 6 h and productivity markedly increased after 8 h of treatment. This is the first report of the induction of furanocoumarin biosynthesis by AsA alone and of the detection of furanocoumarin biosynthesis in a root culture system.

Keywords: *Glehnia littoralis*, Apiaceae, organ culture, ascorbic acid, furanocoumarin

Abbreviations:

AsA ascorbic acid

MeJA methyl jasmonate

Many plants of the Apiaceae, Rutaceae and Moraceae families produce furanocoumarins constitutively and in response to various elicitors [1]. The linear furanocoumarins, including psoralen, bergapten and xanthotoxin, can function as phytoalexins and feeding deterrents. They can photoinactivate DNA through the UV-catalyzed crosslinking of strands [1], [2], and also inactivate cytochrome P450 [1], [3]. On account of their photoreactive properties, they are useful as clinical treatments for skin diseases such as leukoderma and psoriasis. The essential oil extracted from the woody plant *Citrus bergamia* has been used as a natural source of furanocoumarins; however, because of its limited availability, biotechnological production has been investigated using *Ruta graveolens* and *Ammi majus* [4], [5], [6], [7]. Similarly, we have induced furanocoumarin production by yeast-extract treatments in cell-suspension and shoot cultures of *Glehnia littoralis*, though not in root cultures [8], [9]. In an investigation to discover the most effective means of inducing furanocoumarin production, we have found that AsA strongly induces furanocoumarin production in organ cultures of *G. littoralis*, but not in undifferentiated cell suspension cultures. The characteristics

of this specific induction, including its dependence on dose and duration, are briefly reported here.

Using undifferentiated suspension cells and differentiated organ (root and leaf) tissues, the effect of ascorbic acid (AsA) on furanocoumarin induction was determined in comparison to that of yeast extract or methyl jasmonate (MeJA), after 24 h treatment (Fig. 1). Yeast-extract treatment induced bergapten in cell suspension cultures of *G. littoralis*, in accordance with our previous report [8], whereas AsA showed only a slight effect when supplied at 20 mM. On the other hand, differentiated organ cultures of both roots and leaves produced surprisingly higher amounts of furanocoumarins, including psoralen, bergapten and xanthotoxin, following AsA treatment at the same concentration. Xanthotoxin production was dominant, followed in order by bergapten and psoralen. While leaf organ cultures also responded to treatment with yeast extract and MeJA, the level of production was much lower than for AsA treatment. In our root-organ-culture system, only AsA was able to work as a furanocoumarin inducer.

The response of furanocoumarin induction to AsA concentration, from 0.2 to 200 mM, was investigated in root- and leaf-organ cultures and revealed that both types of culture produced abundant furanocoumarins when treated with 10-40 mM AsA, but produced only traces or none at all at other concentrations (Fig. 2) (Fig. 3). Root fresh weight was decreased by AsA treatment and the extent of reduction was substantial at 20 mM and more, whereas leaf fresh weight markedly declined only at 5 mM, at which leaf necrosis was observed (Table 1). When the pH of the culture medium was monitored over a 24-h period during these experiments, it was found that the medium pH initially increased after 2-4 h, and then decreased, depending upon the AsA concentration as well as organs (Fig. 1S, Supporting Information). A drastic decrease of medium pH observed after 8 h in the presence of 5 mM AsA seemed to be associated with leaf necrosis.

To determine the length of contact with AsA that was required to induce furanocoumarin production, root cultures were treated with 20 mM of AsA for various periods of time (2-24 h) and then further

incubated without AsA for a total of 24 h incubation. Furanocoumarins were detected after 6 h contact with AsA, but contact 8 h or more strongly increased production (Fig. 4).

AsA is a well-established antioxidant that protects animal and plant cells from hazardous oxidants and radicals. Plants can produce AsA by themselves and AsA is ubiquitously present in plant cells, even in apoplasts [10], [11], [12]. To date, considerable work has been carried out to elucidate the functions of AsA [10], [11], [13], [14], but few reports have appeared on AsA-dependent phytoalexin induction, except for the finding in rice leaves, where AsA enhanced flavonoid phytoalexin production in combination with the bacterial phytotoxin coronatine, although AsA treatment alone had little effect [15]. In this report, we demonstrate for the first time, to the best of our knowledge, that 10-40 mM of AsA alone can work as a powerful elicitor of furanocoumarin production. In addition, this is the first case of furanocoumarin induction in a root culture system. Efforts are now under way to understand the mechanism how AsA can work as a furanocoumarin inducer.

Materials and methods

Cell suspension cultures and organ cultures of *Glehnia littoralis* Fr. Schmidt ex Miquel (Apiaceae) were established and used for stress treatment, as reported elsewhere [8], [9] and in Supporting Information. For stress treatments, yeast extract (Difco Laboratories), autoclaved at 121°C for 15 min, and methyl jasmonate (Sigma), dissolved in DMSO and filter-sterilized, were added to make 10 g/L and 100 µM final concentrations, respectively. Aqueous solutions of ascorbic acid (AsA) sodium salt (Wako Chemicals, Japan) were filter-sterilized just before addition at various concentrations (0.2-200 mM). As controls, the cultures were treated with water or DMSO, as appropriate. Treatment was usually performed for 24 h. To determine the effects of treatment duration in root cultures, roots were treated with 20 mM AsA for 2, 4, 6, 8 or 24 h and the roots were then transferred to AsA-free medium for further incubation for 22, 20, 18, 16 or 0 h, respectively, amounting in each case to a total of 24 h incubation time. After incubation in the dark at 80 rpm at 25°C, cultures were harvested

by vacuum filtration, separated into tissues (including cells) and media, and stored at -20°C until analysis. Furanocoumarin extraction from tissues and cultured medium and its analysis were described previously [8], [9] and are summarized in Supporting Information.

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Figure legends

Table 1: Fresh weight of tissues after 24 h treatment with various concentrations of AsA.

Roots (80-100 mg/flask) cultured for 10 days in MS medium supplemented with IBA (5 mg/mL) and

leaves (180-200 mg/flask) cultured in MS medium overnight were used, respectively, for AsA

treatment. N (= 3) \pm standard deviation.

Organ	AsA (mM)	Fresh Weight (g)	
		Mean \pm SD	Relative growth
Roots	0	0.55 \pm 0.03	100 \pm 6
	5	0.52 \pm 0.04	95 \pm 7
	10	0.49 \pm 0.06	89 \pm 10
	20	0.46 \pm 0.05	84 \pm 9
	40	0.45 \pm 0.05	83 \pm 9
	80	0.46 \pm 0.05	84 \pm 9
Leaves	0	0.37 \pm 0.01	100 \pm 2
	5	0.28 \pm 0.02	76 \pm 7
	10	0.35 \pm 0.09	96 \pm 25
	20	0.40 \pm 0.05	110 \pm 15
	40	0.38 \pm 0.04	105 \pm 12
	80	0.33 \pm 0.04	92 \pm 10

Figure 1: Furanocoumarin production in cell-suspension, leaf-organ and root-organ cultures of *G. littoralis* after various treatments.

Ascorbic acid (AsA) sodium salt, yeast extract (YE) and methyl jasmonate (MeJA) were added to produce 20 mM, 10 g/L and 100 μ M concentrations, respectively (see text). For controls, water or DMSO was added instead. After 24 h treatment, furanocoumarin contents in the culture medium and tissues were analyzed and the total production calculated. Furanocoumarins induced in response to AsA or yeast-extract treatments were detected almost entirely in the culture medium, whereas those induced in response to MeJA treatment remained largely in the tissues. N (= 3) \pm standard deviation.

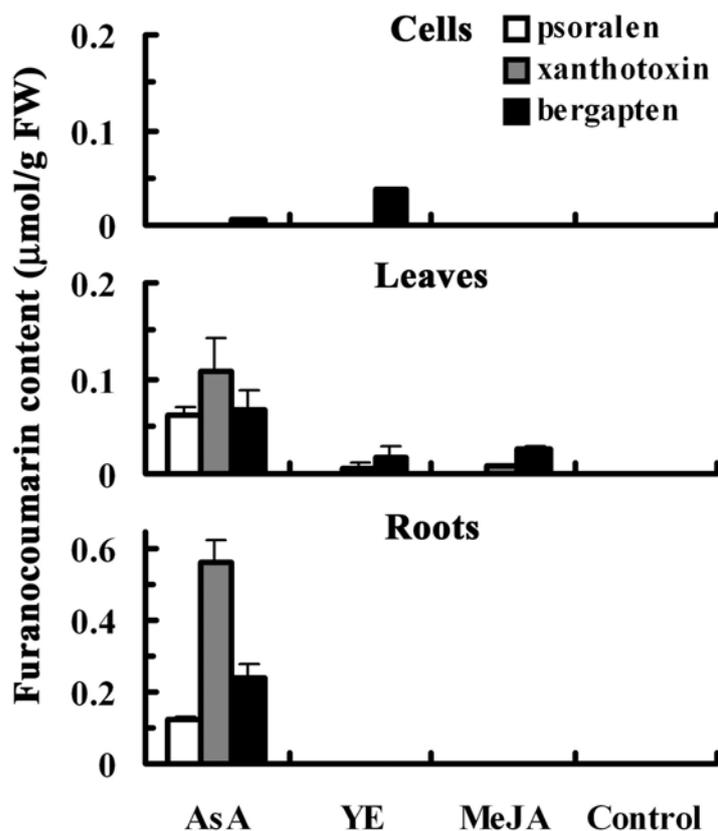


Figure 2: Furanocoumarin production in *G. littoralis* leaf-organ and root-organ cultures treated with various concentrations of AsA.

After 24 h treatment, furanocoumarin production was analyzed. N (= 3) \pm standard deviation.

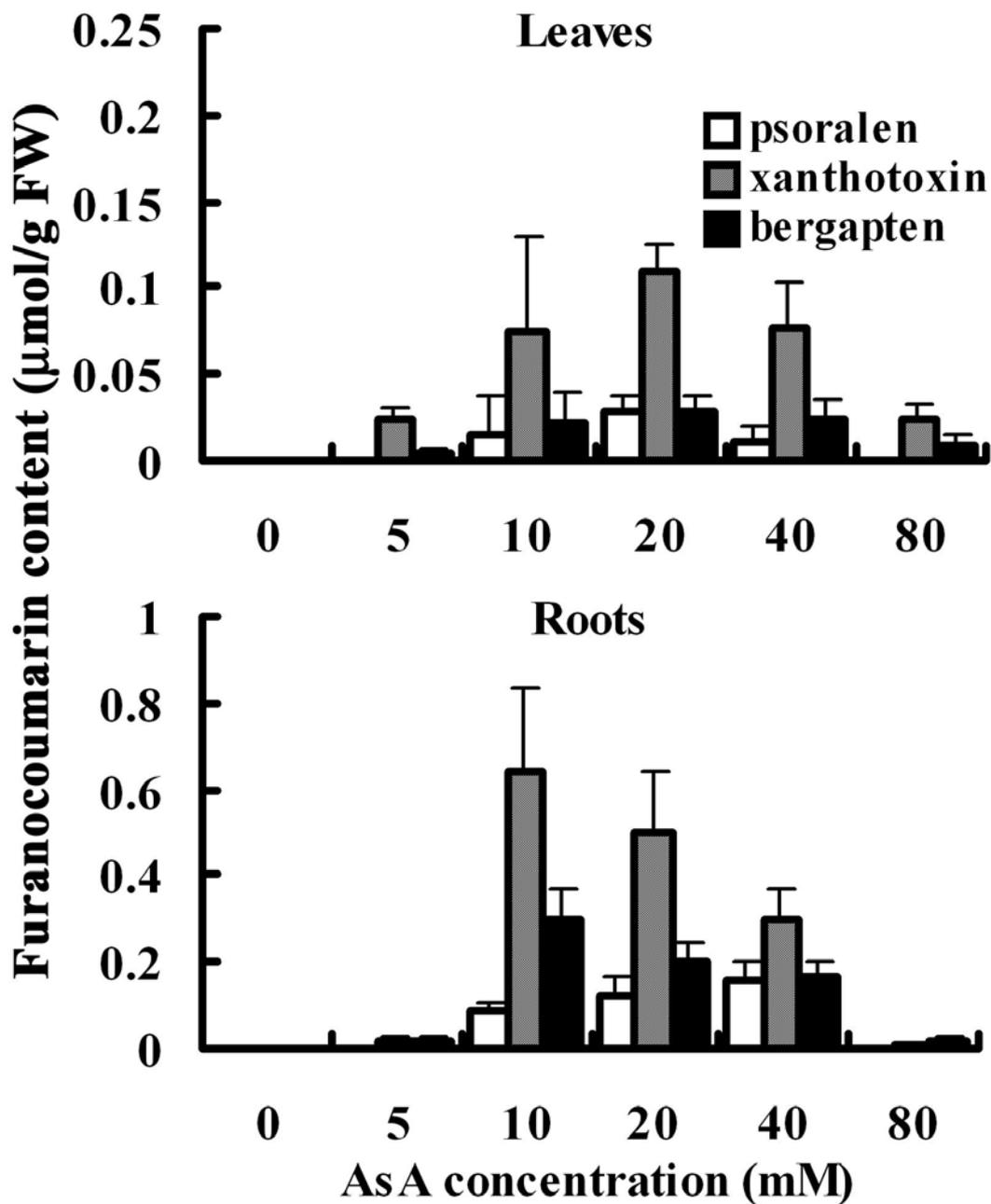


Figure 3: HPLC separation profiles of furanocoumarins in the medium of root-organ cultures of *G. littoralis*.

Culture roots were treated with 10 mM (upper column) and 2 mM (lower column) of AsA for 24 h, respectively. IS, internal standard; Ber, bergapten; Pso, psoralen; Xan, xanthotoxin.

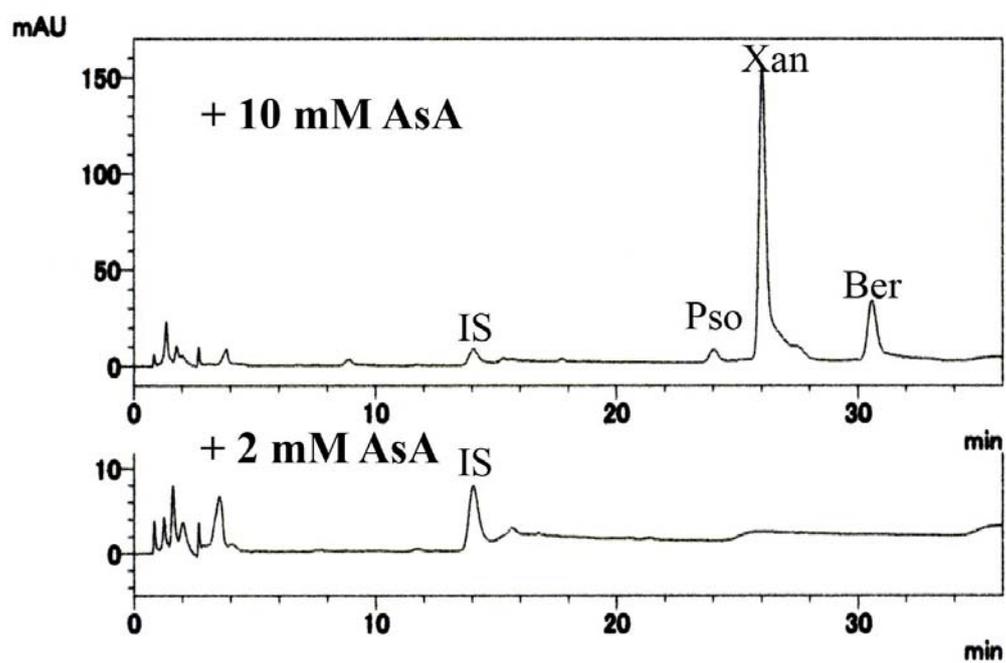


Figure 4: Effect of AsA treatment duration on furanocoumarin induction in root-organ cultures of *G. littoralis*.

Cultured roots were treated with 20 mM of AsA for 2, 4, 6, 8 or 24 h and then incubated further in the medium without AsA for 22, 20, 18, 16 or 0 h, respectively, to give a total of 24 h incubation. N (= 3) \pm standard deviation.

