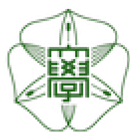
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LETTUCENIN A AS A PHYTOALEXIN OF DANDELION AND ITS ELICITATION IN DANDELION CELL CULTURES

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INTRODUCTION

Many plants, when challenged by microorganisms, produce antimicrobial compounds called phytoalexins[1]. Although the production of phytoalexin has been investigated by numerous researchers, they still dispute whether phytoalexins are effective enough to inhibit pathogens *in vivo*.

Most of researches on phytoalexins have been carried out on species of the Leguminosae, Solanaceae, and other families[2], while the family Compositae — one of the largest families in the plant kingdom — has been relatively neglected[3-7]. We have therefore chosen to study a composite plant, the dandelion (*Taraxacum officinale* Web.), which is indigenous to Europe and has recently been spreading widely in Japan. We are interested in finding out whether plants in this family respond to fungal invasion in the same way as do other plants so far investigated.

We have previously reported[8] a stress-induced antifungal sesquiterpenoid — lettucenin A — which is produced by the dandelion, and since then we have been investigating the induction mechanism of this compound. We here report direct evidence that lettucenin A is indeed active enough to ward off the invasion of a pathogen *in vivo*, and a result which may suggest chemotaxonomical importance of this compound in plants of the Lactuceae.

Since recent reports have indicated that the generation of active oxygen has a part to play in the production of phytoalexin[9-11], we are also prompted to investigate whether active oxygen is involved in lettucenin A production. We established a liquid cell culture of dandelion to conduct this investigation. We report also on the effects of biotic and abiotic stresses on the production of

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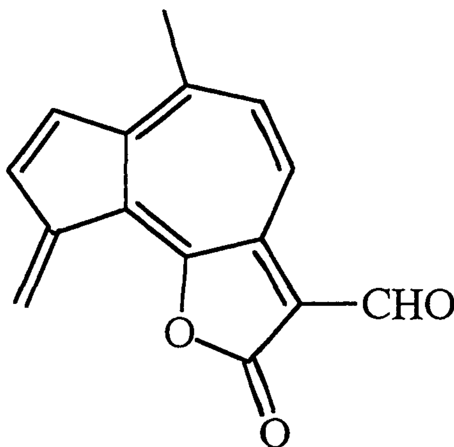
lettucenin A, and adduce results which link the generation of active oxygen species with the lettucenin A production in the liquid cell cultures of *T. officinale*.



Lettucenin A

RESULTS AND DISCUSSION

Lettucenin A as a phytoalexin of dandelion and other composite plants

Lettucenin A was first reported to be a sesquiterpenoid phytoalexin of lettuce by Takasugi *et al.*[3], and found to be produced by dandelion leaves in response to cupric chloride stress[8]. But there was no evidence to show whether this compound is active enough in the leaves to inhibit the invasion of pathogens. Figure 1A shows necrotic spots on a dandelion leaf infected by a pathogenic fungus. Yellow fluorescent rings around the necrotic spots were observed when the leaf was viewed under UV 365 nm light (Fig. 1C). The fluorescence was similar to the one exhibited by lettucenin A, and the presence of this compound in these rings was confirmed by analyzing the ethyl acetate extract of these brown spots by HPLC and TLC bioautography, using *Cladosporium herbarum* as a test fungus[12]. The concentration of lettucenin A (16.4 μg/g fr. wt.) was calculated by HPLC analysis of the ethyl acetate extract, and the amount seemed to be sufficient to inhibit the growth of pathogens, since the growth of *C. herbarum* was completely inhibited at a level of 6.25 $\mu g/cm^2$ on TLC plates (0.25 mm thickness)[8]. The fluorescence of lettucenin A around a necrotic spot in which the pathogen was assumed to be dead (Fig. 1B) was weak (Fig. 1D). In addition, another fluorescent spot was observed under UV light (Fig. 1D) independently of any apparent necrosis (Fig. 1B). This observation suggests that the production of lettucenin A starts at a very early stage of the fungal infection, before the appearance of

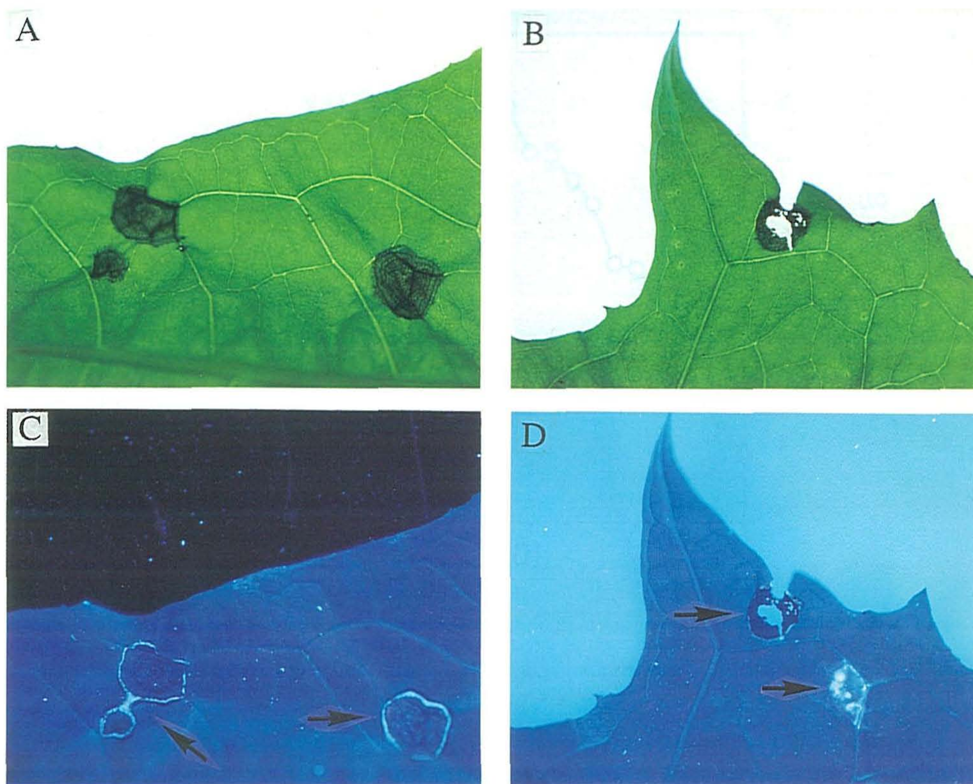


Fig. 1. *T. officinale* leaves infected with a pathogenic fungus. Pictures A and B were taken under white light, C and D under UV 365 nm light. Yellow fluorescent rings and spots can be observed around the infected site.

symptoms, and ends soon after the death of the pathogen. These results show that lettucenin A works effectively to ward off the invasion of pathogens *in vivo*. Since, as we shall mention later, lettucenin A is produced by dandelion cell cultures when they are exposed to a fungal elicitor, it is possible to categorize the compound as a phytoalexin of the dandelion.

Compositae are classified into two subfamilies : Asteroideae (major) and Lactucoideae (minor)[13]. The latter subfamily is composed of 8 tribes, 440 genera and *ca* 8200 species, and characterized by the production of latex. *T. officinale* belongs to the tribe Lactuceae which consists of 70 genera and 2300 species. We examined the production of lettucenin A by some plants in this tribe. The detection of lettucenin A was carried out by TLC autobiography. The following 6 species of plants in 4 genera, when stressed with cupric chloride, produced the compound : *Taraxacum hondoense, Lactuca dentata, L. scariola, Sonchus oleraceus, S. asper* and *Ixeris repens*. Four other species however, did not produce the compound : *Picris hieracioides, Hieracium aurantiacum, H. umbellatum* and *Hypochoeris radicata*. Lettucenin A seems to be a common compound designed to

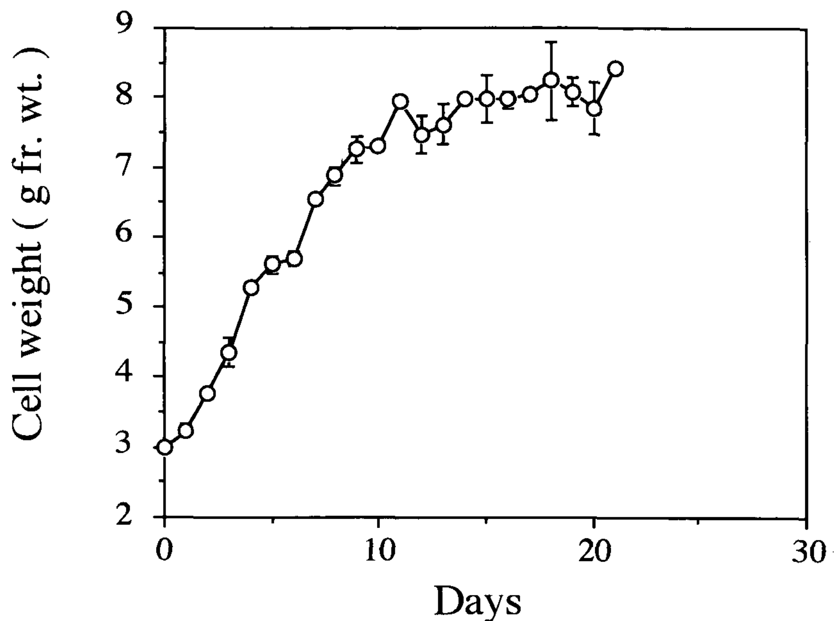


Fig. 2. Growth of *T. officinale* liquid cell cultures. Each value is an average weight of two cultures. Error bars indicate s.d.

withstand fungal invasion in some genera of the tribe Lactuceae, and could be used as a taxonomic characteristic of the plants in this tribe.

Growth of dandelion cell culture

The cell cultures of dandelion used in this experiment were raised from the leaves. They were green and formed aggregates of *ca* 1-5 mm in diameter. Cell cultures (3 g) in 20 ml Gamborg's B5 medium[14] containing 0.5 ppm of naphthaleneacetic acid (NAA) and 1 ppm of benzyladenine (BA) as described in the experimental section grew up to 8 g in 12 days, and kept this weight (Fig. 2) for 21 days. Hook *et al.*[15] have reported that a dandelion cell culture generated from seeds entered the log phase of growth on day 8, and yielded a biomass of 115.3 g fr.wt/l after 24 days of incubation in modified Gamborg's B5 medium. Our green cell cultures raised from leaves did not exhibit the apple-like odor which was released by the beige-color cell cultures reported by Hook *et al.* We also obtained a beige-color culture by incubating the green culture in the dark, which revealed also odorless.

Lettucenin A production

When we stimulated the culture with the elicitor from *C. herbarum*, lettucenin A production occurred within 1 hr and reached a maximum in 2-6 hr (Fig. 3). A similar time course production of lettucenin A took place in cell cultures stressed

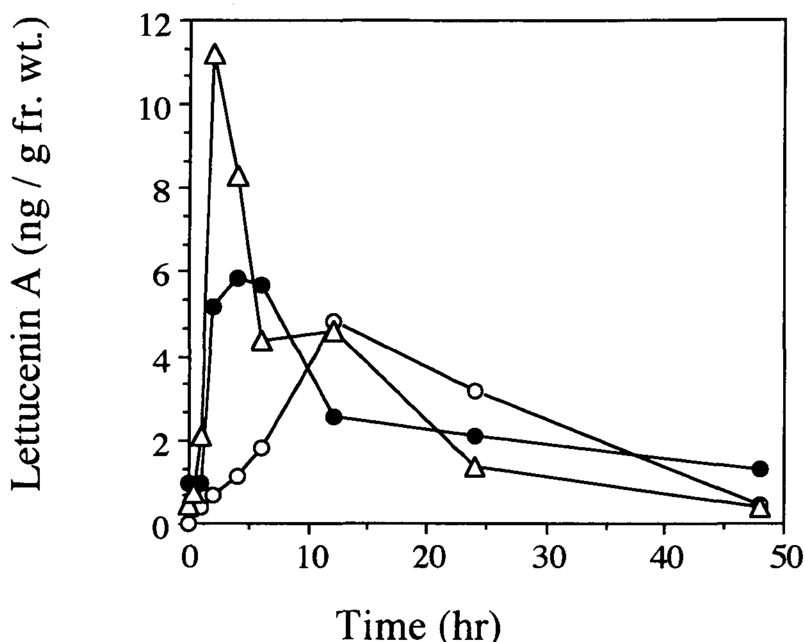


Fig. 3. Time course production of lettucenin A by *T. officinale* liquid cell cultures stressed with —●— : elicitor from *C. herbarum*, —○— : UV light irradiation for 20 min ; and —△— : Cupric chloride (1 mM).

with 1 mM cupric chloride. Thus, the production of lettucenin A in the dandelion cell culture is a remarkably rapid response, when compared with the production of other phytoalexins. For example, the accumulation of glyceollin starts at 6 hr after elicitation[16]. On the other hand, it took 12 hr for the cell cultures stressed with UV light irradiation (Fig. 3) to accumulate lettucenin A at a maximum level. There seems to be some different induction mechanisms for production of lettucenin A. As lettucenin A was not detected in cell cultures which were extracted with ethyl acetate 2 hr after being ground in a mortar, we can deduce that this compound is not derived from a precursor such as its glucoside by the action of endogenous enzymes, since grinding the cells breaks the cellular compartments and would put enzymes and substrates in contact. Although nothing changed in the appearance of cells treated with either the elicitor or UV within 24 hr, both cells and medium treated with cupric chloride turned brown in about 4 hr. Because the amounts of lettucenin A obtained from the medium of stressed cell cultures (5-12 ng/g fr. wt) was 3 or 4 orders of magnitude smaller than that produced in the leaves treated with cupric chloride (3 μg/g fr. wt[8]) or that determined in the naturally infected leaves as mentioned above (16.4 μg/g fr. wt), the productivity of lettucenin A in the cell cultures was very low.

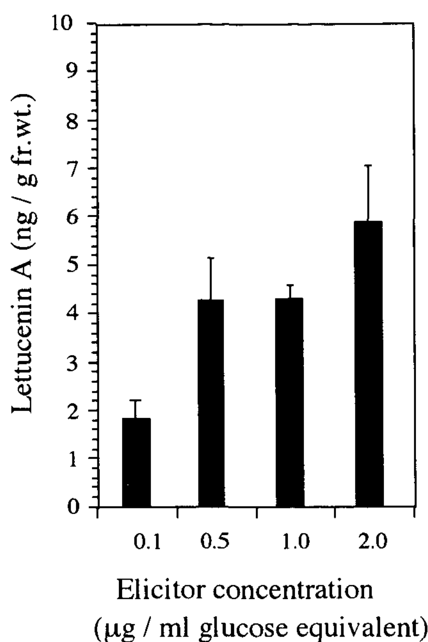


Fig. 4. Lettucenin A production by *T. officinale* liquid cell cultures treated with elicitor from *C. herbarum*. Error bars indicate s. d.

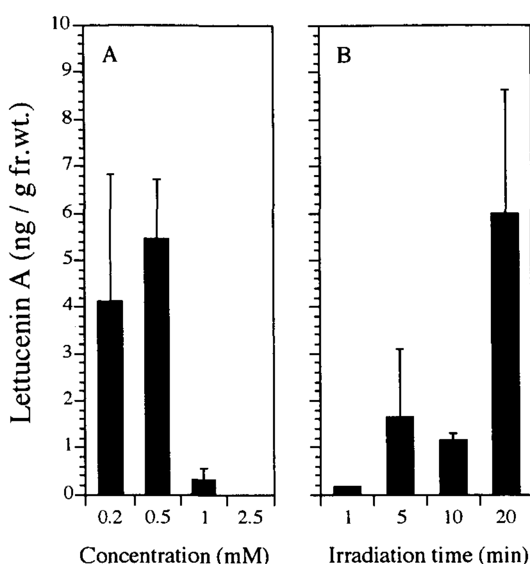


Fig. 5. Lettucenin A production by *T. officinale* liquid cell cultures treated with abiotic elicitors, A : Cupric chloride, and B : UV irradiation. Error bars indicate s. d.

Effects of elicitors, cupric chloride and UV light on the production of lettucenin A

Although there was a significant difference in the amount of lettucenin A produced in the cell cultures treated with 0.1 μg/ml and 0.5 μg/ml glucose equivalent of the elicitor from *C. herbarum*, there seems no remarkable increase in the phytoalexin production in the cultures treated with further amounts of the elicitor (Fig. 4), nor was there any difference in phytoalexin production between the cultures treated with 0.2 and 0.5 mM cupric chloride (Fig. 5). The low or null production of lettucenin A by the cell cultures treated with cupric chloride at the concentration of 2.5 mM and 5 mM can probably be explained by the apparent cellular death provoked by the high concentration of cupric chloride. In the case of UV light stress (radiation of 254 nm UV light, 0.29 mW/cm^2), the production of lettucenin A increased with the length of irradiation time (1-20 min).

Effects of SOD, catalase and ascorbic acid

The addition of SOD, catalase, and SOD+catalase had no noticeable effect on the phytoalexin production induced by the fungal elicitor and the cupric chloride (Fig. 6). Under UV stress, however, those additional treatments caused reduction effects in the following order : SOD<catalase<SOD+catalase (Fig. 6). Although, 10 mM of ascorbic acid (AsA) alone induced the production of

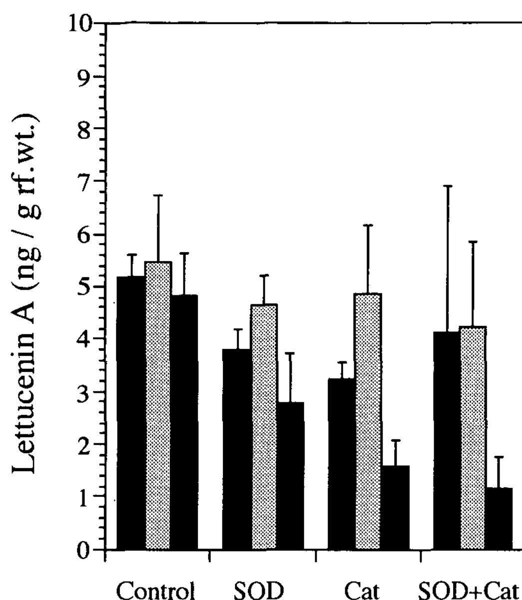


Fig. 6. Effects of SOD and catalase on the production of lettucenin A induced with ■ : elicitor from *C. herbarum*, ▒ : cupric chloride (1 mM), and ■ : UV light irradiation for 20 min. Each enzyme (50 units/ml) was administrated to the *T. officinale* liquid cell culture, and no enzyme was added for contorl. Error bars indicate s. d.

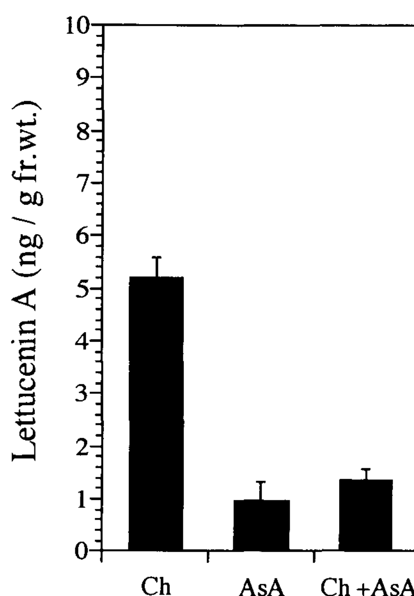


Fig. 7. Effects of ascorbic acid on the production of lettucenin A. Ch : stressed with elicitor from *C. herbarum*. AsA : 10 mM of ascorbic acid alone. Ch+AsA : stress with elicitor from *C. herbarum* in the presence of 10 mM of ascorbic acid. Error bars indicate s. d.

lettucenin A, it also reduced the production induced by the *C. herbarum* elicitor (Fig. 7). Neupane and Norris reported that AsA showed an analogous effect to elicitation by herbivore's grazing on *Glycine max*[17]. Our data is consistent with their observation.

Lettucenin A was also induced in small amounts (*ca* 1 ng/g fr. wt) by other stresses, such as 1 mM *t*-butyl hydroperoxide, and 25 μM calcium ionophore A23187. A cell culture exposed to ischemia by blowing argon gas into a culture flask for 60 min, followed by reperfusion of air, also produced a small amount of lettucenin A. But paraquat (1 mM) which is believed to produce the radical by depriving an electron from the electron transport system in photosynthesis did not induce lettucenin A after either 2 or 24 hr of incubation.

AsA can scavenge radicals but, at high concentrations it may also cause the generation of radicals, since it can take the form of a radical called monodehydroascorbate (MAD) which is highly reactive. We therefore suggest that some kinds of free radical species such as superoxide anion, which can be extinguished by AsA, are involved in the production of phytoalexin and that, at the same time,

free radicals generated from a high concentration of AsA may provoke the production of lettucenin A. These speculations are also supported by the fact that a kind of active oxygen species, *t*-butyl hydroperoxide, also induced the production of lettucenin A. Matsuo *et al.* have reported that exposure to the compound caused serious membrane damage caused in red beet discs[18] and mitochondria from potato[19]. Li *et al.*[20] reported the induction of phytoalexins of rice stressed with linoleic and linolenic acid hydroperoxides. These pieces of data seem to show that membrane damage provoked by free radicals is associated with the production of phytoalexins. Ischemia-reperfusion, a treatment which generates active oxygen species in animals[21], also induced the production of lettucenin A. Van Toai and Bolles.[22] reported that in soybean seedlings about 50 % of root tips were killed, vigor was reduced and the production of superoxide anion was enhanced by reperfusion (postanoxic) injury. Since we found that SOD together with catalase reduced the phytoalexin production induced by UV light irradiation, we deduced that oxygen radicals might be involved in the phytoalexin production of dandelion cells stressed with UV light. We have no explanation, however, for the fact that SOD or catalase had no reducing effects on phytoalexin production induced by the fungal elicitor or cupric chloride.

The beige-color cells grown in the dark, which did not develop chlorophylls, also produced lettucenin A when treated with the elicitor from *C. herbarum*. The result suggests that the development of chloroplasts is not essential to the production of lettucenin A, and that photosynthetic systems are not directly related to the production of phytoalexin. This hypothesis was also supported by the fact that paraquat did not induce the production of lettucenin A.

EXPERIMENTAL

Preparation of *T. officinale* liquid cell cultures

T. officinale calli were raised from the leaves on a Murashige and Skoog (MS) medium[23] containing 1 ppm NAA, 1 ppm BA, 3 % sucrose and 0.2 % gellan gum, and maintained by transferring them onto a fresh medium every 2 weeks at 26 °C under 16 hr light (14.8 w/cm^2) and 8 hr dark period. To make the suspension cultures, the calli were transferred into 20 ml of the MS liquid medium except the gellan gum, in 100 ml Erlenmeyer flasks on a rotary shaker (100 rpm). The cells remained aggregated in this medium, and were maintained by transferring them into a fresh medium every 2 weeks. After 13 months, the calli were transferred into Gamborg's B5 medium[14] containing 0.5 ppm NAA, 1 ppm BA and 3 % sucrose. The cells showed better growth in this medium. When the cells were incubated in 500 ml Erlenmeyer flasks containing 140 ml of the medium, the calli disaggregated gradually over about 6 weeks. Finally, *T. officinale* liquid cell cultures of 1-5 mm size were obtained. The growth of dandelion cell cultures was monitored by measuring fresh weights of the cultures. The

monitoring was started from 3 g of cell cultures in 20 ml of B5 media and continued for 3 weeks. Fresh weights of the cultured cells were obtained after being filtered with suction.

Preparation of crude elicitors

Cladosporium herbarum was incubated in YPD medium (yeast extract 10 g, peptone 20 g, dextrose 20 g in 1 liter of distilled water) for 6 days at 25 °C. The crude elicitor from the mycelia was prepared according to a previously described method[24].

Enzymes and chemicals

SOD (EC 1.15.1.1) from horseradish and calcium ionophore A23187 were obtained from Sigma. Catalase (EC 1.11.1.6) was purchased from Wako Pure Chemical Industries, Ltd. The MS and Gamborg's B5 salt mixtures were purchased from ICN Biochemicals Inc. USA. Paraquat was a kind gift from Nihon Noyaku Co., Ltd.

Treatments of cell cultures with elicitors and other agents

Cultured cells (5 g) transferred into B5 medium (20 ml) and incubated for 1 week were used to determine the amount of lettucenin A. Lettucenin A in the incubation medium was analyzed 2 hr after treatment of dandelion cell cultures with each reagent and stress (except during the time course and UV light irradiation experiments) described below. The amounts of lettucenin A were obtained from three cell cultures and averaged.

The crude elicitor (10 mg) was suspended in 10 ml of distilled water and heated at 120 °C for 4 hr. The supernatant of the suspension (0.1-2.0 ml) was added to the cell culture as an elicitor. The amount of carbohydrate in the elicitor soln. was 20 μg/ml by glucose equivalent. Cupric chloride stress was applied to each of three cultures grown in 20 ml of medium by adding 5 ml of 1, 5, 12.5 and 25 mM of cupric chloride solution. Cultured cells spread on a Petri dish were stressed by irradiation with a sterile UV light (254 nm, 0.29 mW/cm^2) in a clean bench for a set time (1, 5, 10 or 20 min) and were returned to an Erlenmeyer flask to incubate for 12 hr. The effects of SOD and catalase (50 units/ml) were determined by adding 1 ml of 1,000 units/ml enzyme soln. to the medium before the addition of biotic and abiotic stresses. Five ml of paraquat (5 mM), *t*-butyl hydroperoxide (5 mM) and ascorbic acid (50 mM) were added to the medium to determine the effect of each reagent at concentrations of 1, 1 and 10 mM respectively. The effect of calcium ionophore was determined at a concentration of 25 μM by adding 100 μl of a 5 mM EtOH soln. to the medium.

HPLC analysis of lettucenin A

One-week old cultures were treated with elicitors or other reagents. After

each treatment time, the cultured cells were suction-filtered through a cotton plug. The filtrate was extracted with 25 ml of ethyl acetate. The ethyl acetate extract was concentrated were under reduced pressure to *ca* 0.5 ml and subjected to preparative TLC on Silica Gel 60 F_{254} plates (Merck, *Rf* 0.5 in hexane-benzene-acetone=2 : 2 : 1) to purify lettucenin A, which was eluted with 2 ml of ethyl acetate from the silica gel layer corresponding to the relevant band. The eluate concentrated under reduced pressure was dissolved in 0.5 ml of CH_3CN, and subjected to HPLC analysis. Lettucenin A was detected by absorbance at 446 nm using an Inertsil ODS-2 column (4.0×150 mm, GL Science Co., Ltd.) equipped with a 4.0×10 mm precolumn and a solvent system of CH_3CN-H_2O=3 : 2 (retention time 3.40 min) at a flow rate of 1 ml/min. The amount of lettucenin A was calculated on the basis of its molar extinction coefficient ϵ=32000 at 446 nm.

SUMMARY

Letucenin A, a phytoalexin of lettuce (*Lactuca sativa*) and a stress compound of dandelion (*Taraxacum officinale*), was found to be produced in the leaves of the following 6 species (Compositae-Lactucoideae: tribe Lactuceae) when stressed with cupric chloride: *Taraxacum hondoense, Lactuca dentata, L. scariola, Sonchus oleraceus, S. asper* and *Ixeris repens.* Furthermore, lettucenin A was recognized to be working effectively to ward off a pathogenic fungus *in vivo*. We established a liquid cell culture to investigate the mechanisms of phytoalexin production in the dandelion. A biotic elicitor (fungal elicitor) from *Cladosporium herbarum* as well as abiotic stresses (cupric chloride and UV light irradiation) were applied to the culture. The biotic elicitor and cupric chloride induced the production of lettucenin A within 2 hr, while UV light irradiation did within 12 hr. Although SOD (superoxide dismutase) and catalase exhibited no effect on the production of lettucenin A induced by the biotic elicitor or cupric chloride, both enzymes nonetheless reduced the production of lettucenin A induced by the UV stress. Ten mM of ascorbic acid induced the production of a small amount of lettucenin A, but also reduced the production induced by the fungal elicitor. Other stresses, 1 mM *t*-butyl hydroperoxide, 25 μM calcium ionophore A23187 and ischemia-reperfusion treatment, also induced synthesis of lettucenin A, whereas paraquat (1 mM) resulted in no remarkable effect.

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