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Original article

# Ethylene induced shikonin biosynthesis in shoot culture of *Lithospermum erythrorhizon*

Kaori Touno [a,*], Jin Tamaoka [a], Yuko Ohashi [b], Koichiro Shimomura [a]

[a] *Plant Regulation Research Center, Toyo University 1-1-1, Izumino, Itakura-machi, Oura-gun, Gunma 374-0193, Japan*
[b] *National Institute of Agrobiological Sciences Kannon-dai, Tsukuba, Ibaraki 305-8602, Japan*

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**Abstract**

*Lithospermum erythrorhizon* shoots, cultured on phytohormone-free Murashige and Skoog solid medium, produced shikonin derivatives, whereas shoots cultured in well-ventilated petri dishes, produced small amount. Analysis by gas chromatography revealed the presence of ethylene in non-ventilated petri dishes where the shoots, producing shikonin derivatives, were cultured. Therefore, the possible involvement of ethylene in shikonin biosynthesis of shoot cultures was investigated. Treatment of ethylene or the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, resulted in increasing shikonin derivatives contents in cultured shoots. Silver ion, an ethylene-response inhibitor, or aminoethoxyvinylglycine, an ethylene biosynthesis inhibitor, decreased production of shikonin derivatives in cultured shoots. Our results indicate that ethylene is one of the regulatory elements of shikonin biosynthesis in *L. erythrorhizon* shoot culture.


*Keywords:* Ethylene; *Lithospermum erythrorhizon* shoot culture; Shikonin derivatives

## 1. Introduction

The roots of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) accumulate shikonin derivatives, which are red naphthoquinone pigments showing antibacterial [20], wound-healing [7] and anti-tumor activities [14]. It has been reported that the biosynthesis and its regulation of shikonin derivatives in cell suspension culture of *L. erythrorhizon* were influenced by various parameters, such as ammonium ion [5], glutamine [26] and light irradiation [19], which inhibited shikonin derivatives formation, whereas $Cu^{2+}$ ion [6], methyl jasmonate [28] and oligogalacturonide [21] induced shikonin derivatives formation. Recently, shoot cultures of *L. erythrorhizon* established by our laboratory were shown to be capable of biosynthesizing shikonin derivatives [23]. Shikonin derivatives formation was observed on the stem of shoots cultured on phytohormone-free Murashige and Skoog (MS) [13] solid medium containing a high concentration of ammonium ion, which was different from result of cell suspension cultures [5].

LeDI-2, showing the strict dark-specific expression was isolated to investigate the regulatory mechanism of shikonin biosynthesis from cell suspension cultures [29]. LeDI-2 was one of the candidates for the regulatory element of shikonin biosynthesis and specifically expressed in the roots of intact plants. The polypeptide sequence presented shared significant similarities with some root-specific polypeptides such as ZRP3 of maize [11]. Northern blot analysis indicated that LeDI-2 was expressed in the stem of shoot cultures when producing shikonin [22]. We therefore suggest that these shoot cultures may be used as an alternative source of plant material for biochemical and molecular genetic studies on shikonin formation.

To clarify the regulatory mechanisms of this new model system of shikonin biosynthesis by shoot culture, effects of various culture conditions on shikonin derivatives formation were investigated [23,24]. To stimulate shoot growth, shoots were cultured in petri dishes equipped with filters for ventilation. This treatment led to a new problem showing much

*Abbreviations:* ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; MS medium, Murashige and Skoog's medium; PAL, phenylalanine ammonia-lyase.

* Corresponding author.
*E-mail address:* kaori701ym@toyonet.toyo.ac.jp (K. Touno).

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less formation of shikonin derivatives compared with the shoot cultures in non-ventilated petri dishes. This phenomenon was thought to be closely related to the volatile compound(s) released from shoots cultured in non-ventilated petri dishes. There is a possibility that these volatile compound(s), such as jasmonic acid, its derivatives and ethylene, which involved in the general signal transduction network that induces enzymes leading low molecular weight defense compounds [4,28], may induce shikonin derivatives biosynthesis on the shoots cultured in petri dish. It has been reported that methyl jasmonate induced shikonin production in cell cultures [28]. However, there have been no reports of induction of shikonin derivatives by ethylene in *L. erythrorhizon* cultures.

In order to investigate whether ethylene which generated in in vitro involved in induction of shikonin derivatives, we examined the effects of ethylene and ethylene inhibitor on shikonin derivatives formation in *L. erythrorhizon* shoot culture.

## 2. Results

### 2.1. Determination of ethylene emission in shoot culture

We have previously reported that the shoots cultured in the well-ventilated culture vessels, such as petri dish with membrane filter, MilliSeal® (Millipore) and in flasks covered with silicone cap, showed lower content of shikonin derivatives than those cultured in petri dish and in flasks covered with aluminum foil cap [24]. This result indicates that shikonin derivatives formation of in vitro shoot stem was closely related to the air composition within the culture vessel changed by the shoot cultures. It was speculated that the presence of ethylene generated by the shoots was the main cause in the increase in shikonin derivatives formation. In a preliminary experiment, detection of ethylene generated by the shoots cultured in the petri dishes was carried out. Ethylene production and shikonin derivatives formation were quantified by GC and photometer, respectively, at weeks 2 and 4 after shoots were inoculated into the petri dishes with or without membrane filter. Ethylene emitted from the cultured shoots was detected in petri dish without MilliSeal®, and the amount of ethylene and shikonin derivatives increased throughout the culture period (Fig. 1A). In contrast, ethylene did not accumulate in ventilated petri dishes, and only a trace amount of shikonin derivatives was detected (Fig. 1B). There was a direct correlation between ethylene production and shikonin derivatives formation.

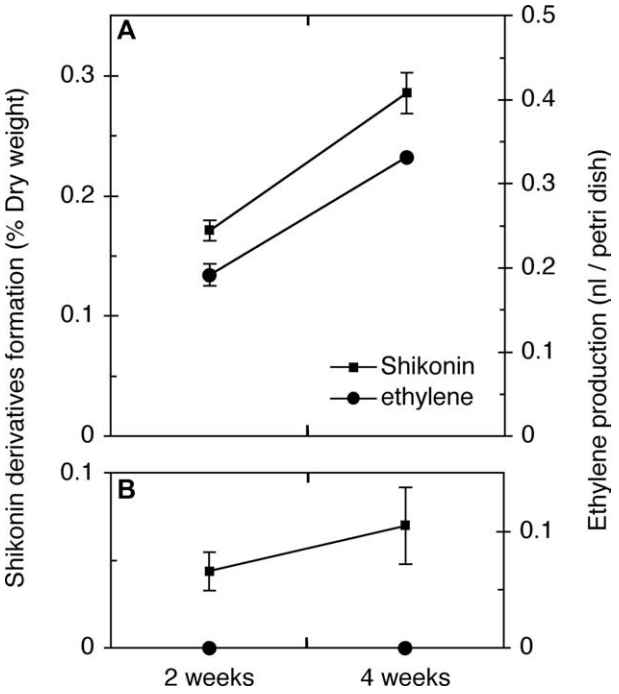


Fig. 1. Time course of ethylene and shikonin production in shoots cultured with (A) or without filters (B). A, two shoots (ca. 4 cm in length) were cultured on MS solid medium (petri dish) at 25 °C for 4 weeks in the dark. B, two shoots were cultured in petri dish with three holes (ca. 5 mm φ) sealed with membrane filters (Millipore)/a cover under the same culture condition as mentioned in A. Error bar: S.D. ($n = 4$).

### 2.2. Effect of ethylene and ACC on shikonin derivatives formation in shoot cultures

In order to clarify the involvement of ethylene in shikonin derivatives formation, effects of ethylene and ACC on shikonin derivatives formation in shoot cultures were investigated. The shoots cultured in the petri dish were exposed to two different concentrations of ethylene (10 nl and 10 μl per petri dish). The shoots treated with ethylene showed higher shikonin derivatives content than those untreated. Especially, treatment of 10 μl ethylene per petri dish enhanced shikonin derivatives formation about threefold compared with the untreated shoots (Fig. 2).

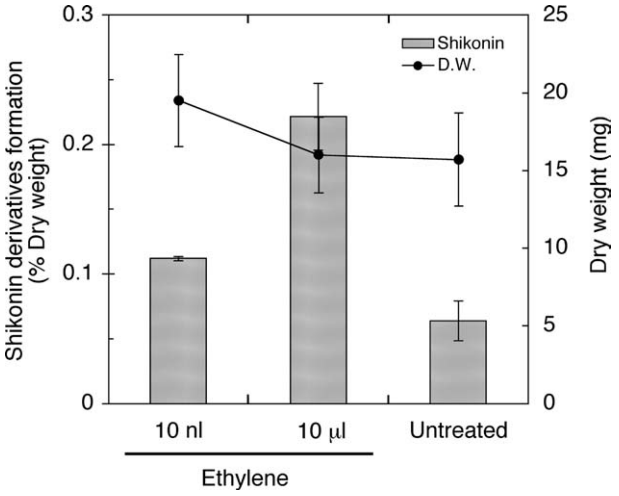


Fig. 2. Effects of ethylene on shikonin production in shoot culture. Two shoots (ca. 2 cm in length) were pre-cultured on petri dish with a hole (ca. 5 mm φ)/a cover sealed with membrane filter on MS solid medium at 25 °C for 1 week in the dark. After pre-culture, ethylene was added to petri dish and the shoots were cultured for 3 weeks after the administration. Error bar: S.D. ($n = 8$).