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Effects of Abiotic Inducers on Sesquiterpene Synthesis in Hairy Root and Cell-Suspension Cultures of *Hyoscyamus muticus*

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ABSTRACT

The effects of hydrogen peroxide (H_2O_2), salicylic acid (SA), ethanol (EtOH), and methyl jasmonate (MJ) on the lubimin and solavetivone production in hairy root and cell suspension cultures of *Hyoscyamus muticus* were investigated. H_2O_2 (0.194–1.94) m*M* did not show any positive effect of the lubimin and solavetivone production. Lubimin production of cell-suspension cultures increased 50% in the presence of 40 μ*M* SA. For hairy root cultures, lubimin and solavetivone production increased 5 and 48%, respectively, in the presence of 4 μ*M* SA •EtOH (20 m*M*) increased the lubimin and solavetivone concentrations 13% for hairy roots suggesting a potential rule for wounding or membrane disruption. MJ (4 μ*M*) and 20 m*M* EtOH increased the lubimin and solavetivone concentrations 25 and 85%, respectively for hairy roots of *H. muticus*.

Index Entries: *Hyoscyamus muticus*; lubimin; solavetivone; cell suspension; hairy root; hydrogen peroxide; salicylic acid; ethanol; methyl jasmonate.

INTRODUCTION

Biologists and chemical engineers have been intensively investigating plant cells that produce various chemicals for the last 30 yr. The plant researchers showed that 30 different types of plant cells produce much more plant chemicals than the natural plants *(1)*. Plants have an ability to

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produce secondary metabolites (phytoalexins) to defend themselves against microbial infection *(2)*. These chemicals accumulate at the site of infection in intact plant *(3)*. Phytoalexins also accumulate after exposing plant tissues to fungal mycelial extracts *(2)*.

Commercialization of chemical production from plant tissue culture depends on finding the appropriate techniques to increase culture productivity. Elicitors are effective in inducing phytoalexin accumulation in cultured plant tissues *(4)*. Crude fungal extracts would be preferred over purified elicitors for an industrial process to reduce elicitor purification costs. However, they may contain a mixture of structurally different components with elicitor-active or elicitor-antagonistic properties. For the investigation of events related to recognition and consequent signal transduction, elicitor purification is needed *(5)*. It is also desirable to minimize the elicitor addition. Induction of the production of secondary metabolites in cultured plant cells by an abiotic elicitor mimics the defensive response of the plants to invasion by pathogenic microorganisms. The recent work suggests that the oxidative burst is not directly involved in phytoalexin formation. Some researchers have found that exogenous application of H_2O_2 is capable of inducing secondary metabolite formation *(6)*. Gustine and Devlin *(7)* observed that H_2O_2 and O_2 are produced during the plant defense against a pathogen, which are not otherwise necessary for phytoalexin production. Injection of tobacco leaves with salicylic acid (SA) (0.01%) prior to the tobacco mosaic virus (TMV) inoculation caused a dramatic reduction in lesion number *(8)*. Klessig et al. *(9)* proposed that salicylic acid is a natural signal molecule for the activation of plant defense responses, inducing systemic acquired resistance, SAR. The research of Mizukami et al. *(10)* showed that a dramatic increase in rosmarinic acid content in cultured cells of *Li erythorhizon* resulted when exposed to methyl jasmonate (MJ). Sanz et al. *(11)* showed that, in olive leaf disks, both 1-aminocyclopropane-1-carboxylic acid content and ethylen-forming enzyme activity were clearly stimulated by 45 μ*M* MJ. Preincubation of suspension-cultured parsley cells with MJ greatly enhances their ability to respond to fungal elicitor by secretion of coumarin derivatives *(12)*.

In this study, the effects of H_2O_2, SA, ethanol (EtOH), and MJ on the sesquiterpene synthesis in hairy root and cell-suspension cultures with or without fungal elicitor of *Hyoscyamus muticus* were investigated.

MATERIALS AND METHODS

Cultures

Cell suspension and soil bacterium *Agrobacterium rhizogenes* transformed hairy root cultures of *H. muticus* were maintained using biweekly subculturing in 50 mL B5 medium (with 0.2 mg/L2, 4-D for cell suspen-

sion) in 125-mL Erlenmeyer flasks. The cultures were incubated on a gyratory shaker with a 2-in. stroke at 100 rpm and 25°C.

Inoculation/Elicitation/Harvest

Experiments were carried out in 125-mL Erlenmeyer flasks containing 50 mL of fresh medium. Each flask was inoculated with 0.25 g fresh weight of cells or roots as described by De Hass *(13)*. After 14 d of growth, 0.5 or 1.0 mL sterile fungal elicitor and abiotic elicitors (H_2O_2, SA, EtOH, MJ) were added after inoculation. The cultures were maintained on a gyratory shaker at 25°C for 24 h. Samples were collected after incubation and analyzed for lubimin and solavetivone. All the experiments were conducted in triplicate.

Preparation of Elicitor

The fungal elicitor was prepared from the soil fungus *Rhizoctonia solani (14)* grown in a 5-L Bioflow III (New Brunswick Scientific, Edison, NJ) in SH medium at 200 rpm with Rushton impellers and no baffles. The elicitor was prepared by resuspending mycelium in Mill-Q water (0.3 g fresh weight/L) followed by homogenization and autoclaving for 3 h. The elicitor consisted of the hydrolyzed supernatant after centrifugation for 30 min at 20,000*g (15)*. The final elicitor had 52 μg glucose equivalent/mL as measured by the anthrone assay *(16)*.

Analysis

Analysis was carried out using HPLC. The induced sesquiterpenes lubimin and solavetivone were extracted by passing 5 mL of cultured medium through a "C18 sep-pak" cartridge (Waters, Milford, MA) and eluted with 3 mL of methanol. The methanol was dried under nitrogen atmosphere, and the residue was resuspended in 2 mL acetonitrile:water (60:40). The chromotographic separation was carried out isocratically on a Waters "μ Bonda pack C18" reverse-phase column. The mobile phase was 60:40 acetonitrile:water at flow rate of 2 mL/min. Solavetivone and lubimin were measured at 200 and 245 nm, respectively.

RESULTS AND DISCUSSION

The effects of H_2O_2 on the solavetivone and lubimin production in cell suspension and hairy root cultures of *H. muticus* are shown in Fig. 1 and 2. H_2O_2 does not have any effect on the sesquiterpene synthesis as observed from the *t*-test results. These are in agreement with the results of Gustine and Devlin *(7)*, who observed that H_2O_2 is produced during the plant defense against the pathogen, but it is not necessary for phytoalexin production.

As can be seen from Fig. 3, 40 μ*M* SA caused a 50% increase in the lubimin production of *H. muticus* cell-suspension culture compared to fungal elicitor. However, 40 μ*M* SA does not have any positive effect on the

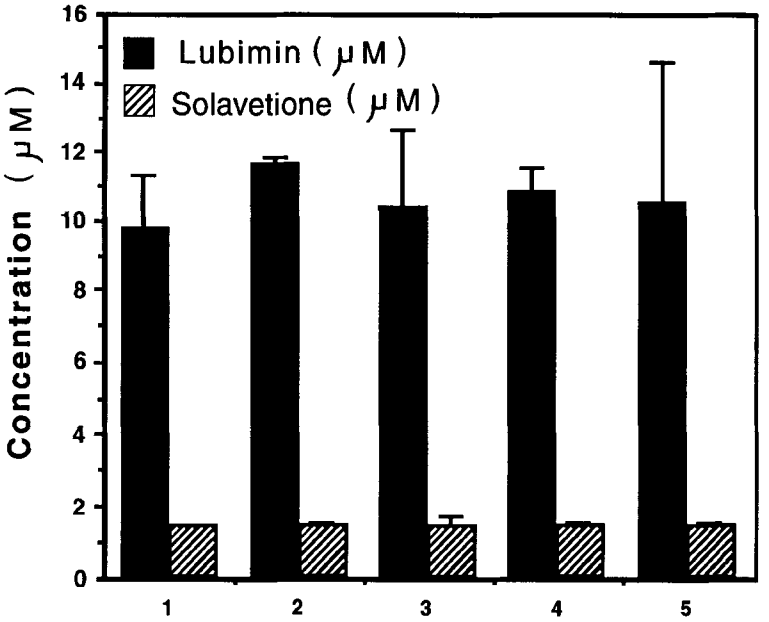

Fig. 1. Effects of H_2O_2 on the cell suspension cultures. 1. 0.5 mL fungal elicitor. 2. 0.5 mL elicitor + 1.94 m*M* H_2O_2. 3. 0.5 mL elicitor + 0.97 m*M* H_2O_2. 4. 0.5 mL elicitor + 0.388 m*M* H_2O_2. 5. 0.5 mL elicitor + 0.194 m*M* H_2O_2.

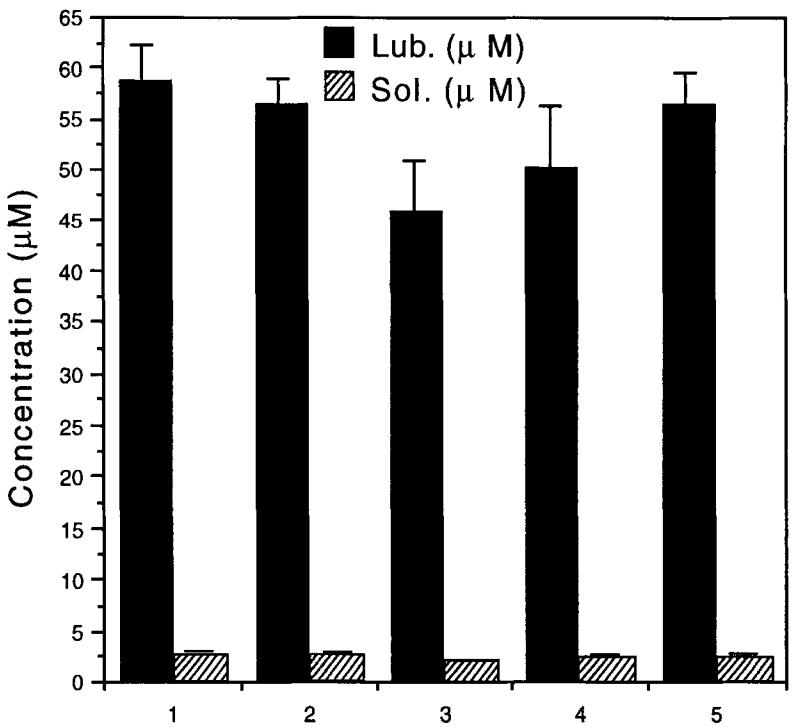

Fig. 2. Effects of H_2O_2 on the hairy root cultures. 1. 0.5 mL fungal elicitor. 2. 0.5 mL elicitor + 1.94 m*M* H_2O_2. 3. 0.5 mL elicitor + 0.97 m*M* H_2O_2. 4. 0.5 mL elicitor + 0.388 m*M* H_2O_2. 5. 0.5 mL elicitor + 0.194 m*M* H_2O_2.

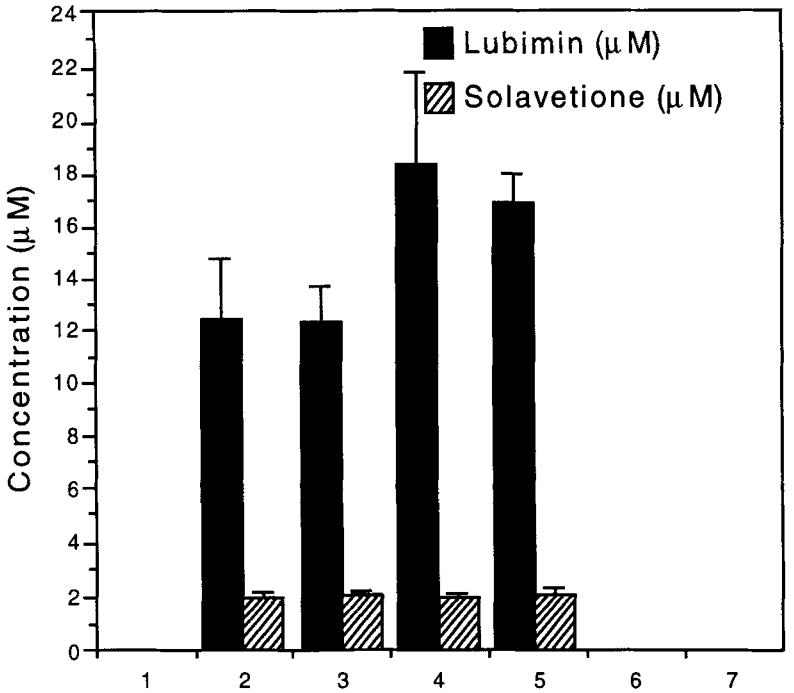

Fig. 3. Effects of SA on the cell-suspension cultures. 1. Control. 2. 0.5 mL fungal elicitor. 3. 0.5 mL elicitor + 0.194 m*M* H_2O_2. 4. 0.5 mL elicitor + 40 μ*M* SA. 5. 0.5 mL elicitor + 0.194 m*M* H_2O_2 + 40 μ*M* SA. 6. 40 μ*M* SA (without elicitor). 7. 0.194 m*M* H_2O_2 + 40 μ*M* SA (without elicitor).

sesquiterpene synthesis of hairy root cultures (Fig. 4). SA (4 μ*M*) increased 5 and 48% in lubimin and solavetivone production, respectively (Fig. 5). Although statistically significant, the effects on total sesquiterpene production are relatively small because solavetivone is present in much lower levels. These results are in agreement with the results of previous researchers *(8,9)*, who claimed that SA is a natural signal molecule for the activation of plant defense responses. It must be indicated that SA does not have any positive effect without the fungal elicitor.

MJ does not appear to have any positive effect on the lubimin production when it is dissolved in water (Fig. 5). For this reason, MJ was dissolved in ethanol. Figure 6 shows the effects of EtOH and MJ. Both of these do not produce sesquiterpene without the fungal elicitor. In the presence of 1 mL of fungal elicitor, 40 μ*M* MJ in 20 m*M* EtOH increased the lubimin and solavetivone concentrations 25 and 85%, repectively, compared to fungal elicitor. This observation supports the results of Kauss et al. *(12)* and Mizukami et al. *(10)*. EtOH alone increased the production of sesquiterpenes as much as 13%.

It can be concluded that SA, EtOH, and MJ appear to facilitate signaling in plants. The interaction of ethanol with the defense response is probably indirect through membrane disruption and is indicative of a

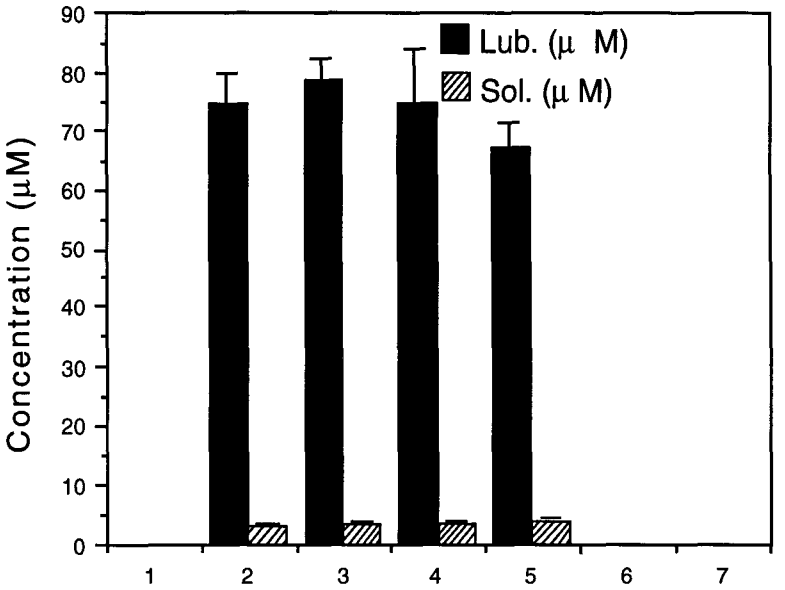

Fig. 4. Effects of SA on the hairy root cultures. 1. Control. 2. 0.5 mL fungal elicitor. 3. 0.5 mL elicitor + 0.194 m*M* H_2O_2. 4. 0.5 mL elicitor + 40 μ*M* SA. 5. 0.5 mL elicitor + 0.194 m*M* H_2O_2 + 40 μ*M* SA. 6. 40 μ*M* SA (without elicitor). 7. 0.194 m*M* H_2O_2 + 40 μ*M* SA (without elicitor).

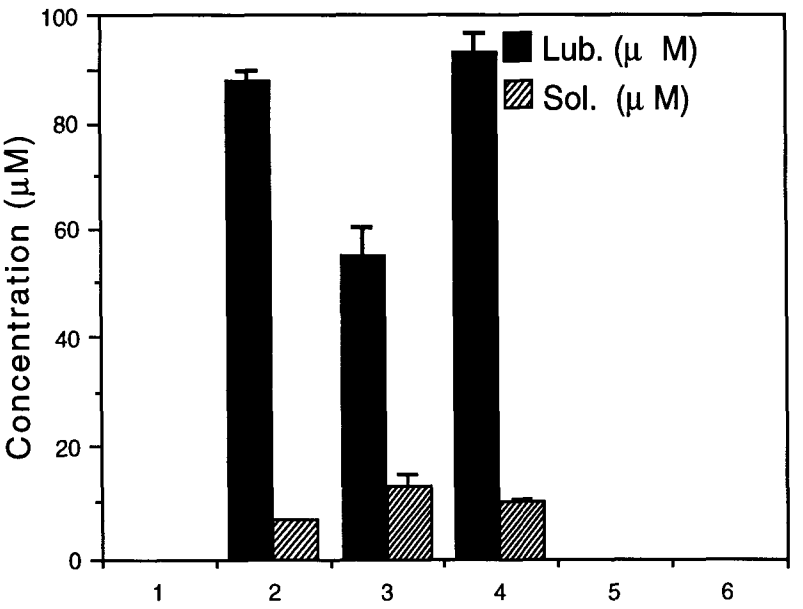

Fig. 5. MJ and SA effects on the hairy root cultures. 1. Control. 2. 1 mL fungal elicitor. 3. 1 mL fungal elicitor + 4 μ*M* MJ. 4. 1 mL fungal elicitor + 4 μ*M* SA. 5. 4 μ*M* MJ (without elicitor). 6. 4 μ*M* SA (without elicitor).

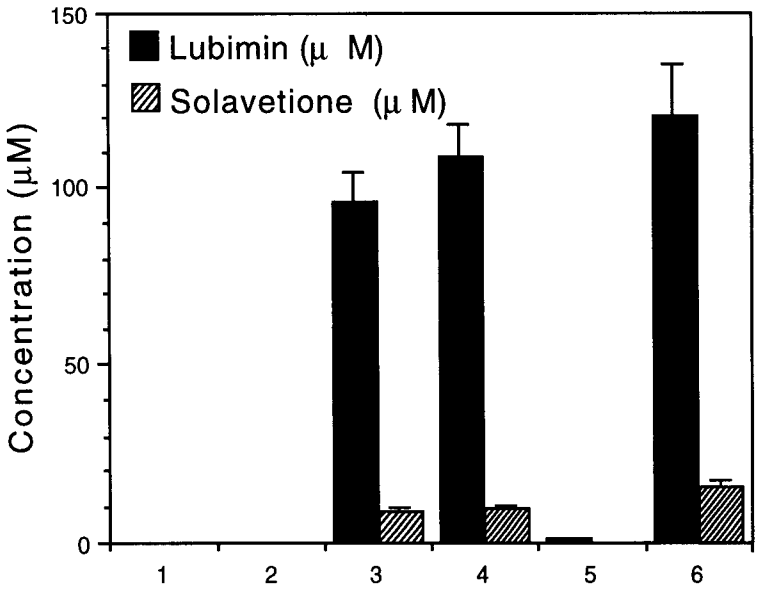

Fig. 6. MJ and EtOH effects on the hairy root cultures. 1. Control. 2. 20 m*M* EtOH (without elicitor). 3. 1 mL fungal elicitor. 4. 1 mL fungal elicitor + 20 m*M* EtOH. 20 m*M* EtOH + 4 μ*M* MJ (without elicitor). 6. 1 mL fungal elicitor + 20 m*M* EtOH + 4 μ*M* MJ.

potential interaction of wounding in this signaling process. Futher studies are needed to characterize effects such as handling of tissue (wounding), gas exchange (ethylene), and interaction of these signals at saturated and subsaturated fungal elicitor response.

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