J. Rubb. Res., **5**(1), 53–63

Wound-induced Differentiation in Hevea brasiliensis *Shoots Mediated by Jasmonic Acid*

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The effects of mechanical wounding on laticifer differentiation in Hevea brasiliensis *were investigated by using the young stems in epicormic shoots. Light wounding by scraping to break the epidermal cells of young stem usually caused the formation of a line of secondary laticifers in the area roughly equal to that of the wound. More laticifers formed in a larger area in response to heavier wounding, such as that caused by deep scraping to remove part of cortex tissues of young stem or by cutting its bark into xylem. The first laticifers induced by scraping and cutting were visible as early as 6 days after wounding. The induction of laticifer differentiation by wounding in young stem was enhanced by the application of jasmonic acid and linolenic acid, whereas induction was hindered to different degrees by the application of diethyldithiocarbamic acid and nordihydroguaiaretic acid, which are inhibitors of jasmonate biosynthesis. Together with other evidence, these observations indicate that laticifer differentiation upon wounding is mediated by jasmonates. The involvement of jasmonates in wound morphogenesis is discussed.*

Key words: laticifer differentiation; wounding; epicormic shoots; jasmonic acid; linolenic acid; diethyldithiocarbamic acid; nordihydroguaiaretic acid; *Hevea brasiliensis*

Wounding is an external stimulus that affects plant development. One can define wounding as a mechanical process which destroys cells in a specific area of tissue and thus the main developmental events caused by wounding are the regeneration of tissue lost by wounding and the formation of protective barriers at the wound surface[1–4]. In addition, wounding may induce the development of particular protective structures, such as the so-called traumatic resin ducts or gum ducts in many conifer trees and woody dicotylidinous plants[5].

The laticifers of *Hevea brasiliensis* may be regarded as another example of wound inducible protective structures. It was assumed that the laticifers have the function of protecting plant from wound stress[5]. The latex in the laticifers may play a role in wound healing, as a defence against herbivore and also perhaps, as a defence against micro-organisms. The assumption of the laticifers as protective structure is supported by the recent discoveries that very high levels of antifungal and antibacterial substances are present in the latex

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from the laticifers (for reviews see d'Auzac *et al.*[6,7]). There is evidence that the formation of the laticifers may be induced by wounding. Our previous data have shown that latex exploitation is one of the most important factors that influence the formation of the laticifers in *Hevea* tree trunk (Hao and Wu)[8]. Compared to the unexploited trees, the exploited trees produced 2–3 times the number of laticifers in the trunk during the course of the experiment. Since latex is exploited by incising the tree bark where laticifers occur, the increase in laticifer number in exploited trees was attributed to mechanical wounding. An extensive study is therefore needed to clarify the role of mechanical wounding in laticifer differentiation.

Recent studies indicate that responses of plants to wounding are regulated by multiple signal transduction pathways in which jasmonates (jasmonic acid and its related compounds) are significant components (Seo *et al.* and Leon *et al.*[9,10]). Jasmonates regulate the expression of plant defence genes in response to various environmental stress including mechanical wounding, and they also play fundamental and remarkable roles in modifying growth and development of plants[11–13]. However, among the developmental events which are known to be modified by jasmonates, none are related to wound morphogenesis. We have recently found that application of jasmonic acid (JA) and linolenic acid (LA), the precursor of JA biosynthesis, induced the differentiation of laticifers in *Hevea* (Hao and Wu)[14].

In the present study, we show evidence that mechanical wounding induces laticifer differentiation and the induction may be regulated by the jasmonate biosynthesis pathway.

MATERIALS AND METHODS

Plant Material

All the wounding experiments, were performed on the epicormic shoots from plants of *Hevea brasiliensis* Mull. Arg. clones RY7-33-97 or RY 88-13 budded on seedlings. The plants were grown in the nursery on the experimental farm of our Academy on Hainan Island, China. They were pruned each year and epicormic shoots grew from the latent buds of the pruned branches. As our previous paper showed (Hao and Wu)[14], the epicormic shoots flush five to six times a year, and such a shoot therefore consists of a series of foliage leaf clusters, separated by leafless lengths of stem. Each of these morphologically distinct growth increments represents a growth flush, and is referred to as an extension unit (*Figure 1*).

Wounding Experiment

Scraping and cutting experiments were performed on the epicormic shoots during April to September, when they had produced two–four extension units. On the top extension unit or the top two extension units, the stem was wounded with a sharp razor by scraping or cutting the area of the stem immediately below the lowest foliage leaf of the extension unit. For scraping experiment, a wound 6 mm × 10 mm rectangular was made by scraping the bark of the stem and for cutting experiment, a wound 10 mm in length and parallel to the stem was made by cutting the bark into xylem of the stem perpendicularly to the stem surface (*Figure 1*). Samples of bark with the wound were excised from the stem, one to 40 days after wounding according to need.

Treatment of Wound with Chemicals

Immediately after wounding, the wound was plastered with lanolin paste containing a test

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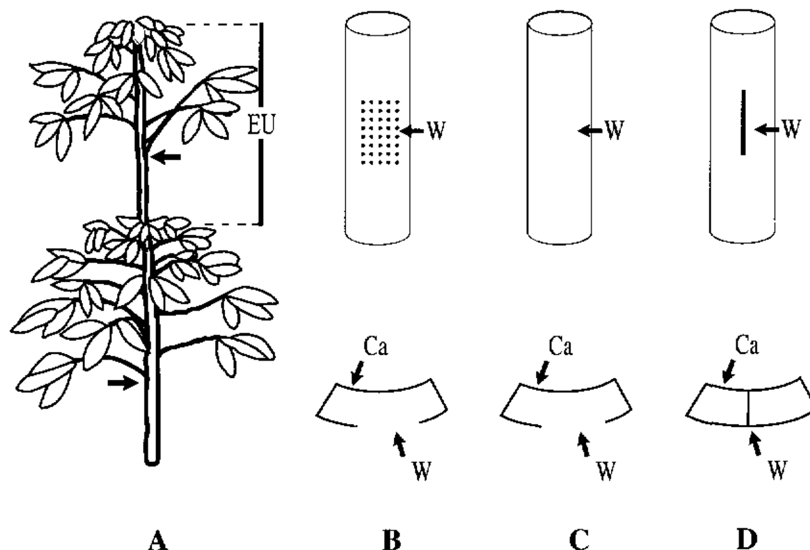

Figure 1 Diagram illustrating the wounding experiment. A, Hevea *epicormic shoot showing the upper most extension unit (EU) and the sites at which the wounds were made (arrows). B–D, Surface view (up) and cross section (down) of the stems showing wounding by scraping to break epidermal cells (B), by scraping to remove epidermal cells and part of cortex tissues (C) and by cutting (D). Ca, cambium. W, wound.*

chemical and covered with a small piece of polyethylene membrane. Each plastering on a wound used about 0.05 g lanolin paste. Samples of the bark with wound were excised 40 days after wounding.

The concentrations of the chemicals were expressed as weight percentages in the lanolin paste containing the chemicals. (±)-Jasmonic acid, linolenic acid, diethyldithiocarbamic acid (DIECA) and nordihydroguaiaretic acid (NDGA) were purchased from Sigma (St. Louis, MO, USA). The high purity Lanolin was from China.

Measurements of Phloem

Sections of bark samples were made (see below) for light-microscopic observation and the phloem tissues in bark cross-sections were measured under a light microscope.

Secondary laticifer number was determined by measuring all laticifers derived from vascular cambium. Data were collected from three randomly chosen sections for each sample. The formation of the secondary laticifers had to be induced by wounding and other treatments since secondary laticifers were never produced during the experiment period under normal conditions in the young stem of the epicormic shoot (Hao and Wu)[14].

Secondary phloem cell layer number was determined by measuring the cell number in a radial cell line in the secondary phloem axial system, including phloem parenchyma cells, laticiferous cells and sieve elements, but no

TABLE 1. SECONDARY LATICIFER DIFFERENTIATION AS INFLUENCED BY BREAKING EPIDERMAL CELLS—DETERMINED 40 DAYS AFTER WOUNDING

Treatment	Secondary laticifer number	Secondary phloem cell layer number
Wounding	198.0 ± 41.0	15.0 ± 0.7
Control	0	15.8 ± 0.9

[a]Results are means ± s.d. of ten shoots.

companion cells. Data were collected from five randomly chosen sites in a section for each sample. This cell layer number expresses the number of times the cambium fusiform initials divided; the division resulting in the formation of the phloem axial system.

Light Microscopy

Bark samples were fixed in 80% ethanol, treated with iodine and bromine in glacial acid[15], and embedded in paraffin after dehydration. Sections were cut with a microtome and stained with Fast Green. The laticifers in sections could be recognised, since the rubber in the laticifers was brown due to the iodine-bromine treatment. As tannin-like substances were eliminated from the samples by the fixation of ethanol, no cells containing tannin-like substances in sections could be recognised.

RESULTS

Laticifer Differentiation Induced by Scraping Stem

Scraping of the young stem of epicormic shoot led to the formation of secondary laticifers that were never observed in unwounded stem (*Figure 2 A–C*). The first induced secondary laticifers were detectable 6 days after scraping. Breaking epidermal cells of stem by scraping caused the formation of a line of secondary laticifer (*Figure 2 B*) in the area roughly equal to that of the wound. In the samples collected 40 days after scraping, the cell layer number of the secondary phloem axial system did not change significantly, while secondary laticifers were induced by wounding in the secondary phloem (*Table 1*). This indicates that the wounding had no effect on the division rate of the cambium fusiform initials.

The severe wounding by deep scraping to remove the epidermal cells and part of cortex tissue induced formation of one or two lines of secondary laticifer. As seen in the cross section of stem, the lines of secondary laticifer had the laticifers with higher density (*Figure 2 C*) and this occurred in an area larger than that of the wound. In this area, the secondary phloem cell layer number decreased (compare *Figure 2 C* with *Figure 2 A*), indicating an inhibition of the division rate of the cambium fusiform initials.

Laticifer Differentiation Induced by Cutting Stem

The young stem of epicormic shoots were wounded by cutting. This led to latex flow as the primary laticifers in the stem were broken. After the latex flow stopped, the wounds were exposed to the air for some stems and for the others, the wounds were plastered with lanolin paste and covered with a small piece of polyethylene membrane. In both kinds of the wounded stem, with and without covering,

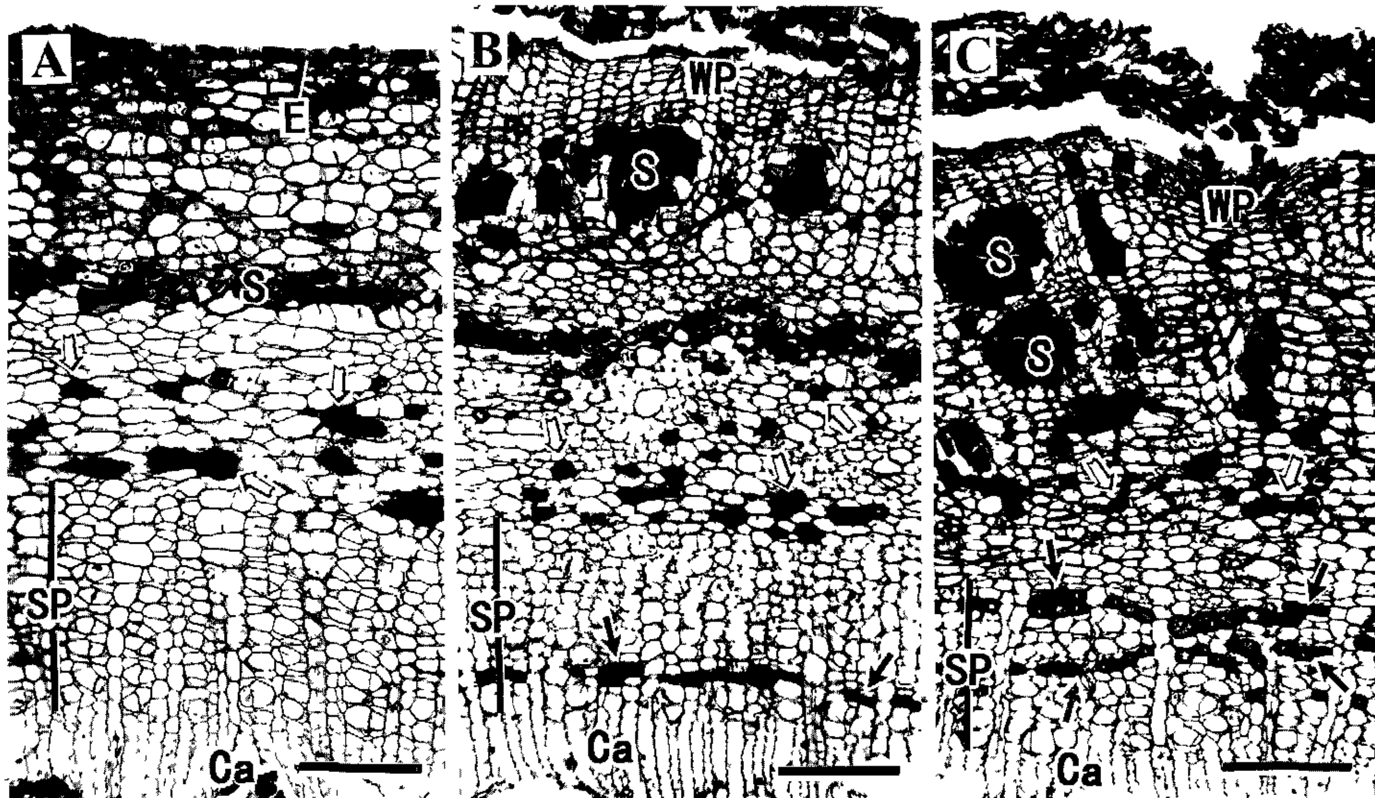

Figure 2. A–C Cross-sections of bark, showing laticifer differentiation as influenced by scraping. Sampling for observation, 40 days after wounding: A, unwounded bark, having no secondary laticifers; B, bark scraped to break epidermal cells, having a line of secondary laticifers (black arrows); C, bark scraped to remove part of cortex tissues, having two lines of secondary laticifers (black arrows). White arrows, primary laticifers; Ca, cambium; E, epidermis; SP, secondary phloem; S, sclereids; WP, wound periderm. Bars = 50 µm.

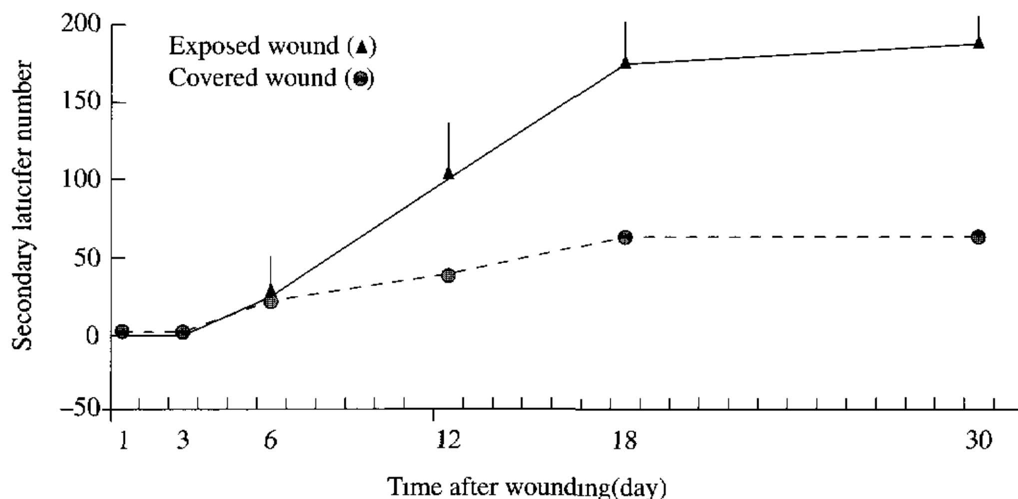

Figure 3 Secondary laticifer differentiation as influenced by cutting (Results are means ± s d of five shoots)

more than one line of secondary laticifer differentiated from the cambial cells around the wound The course of formation of the laticifer was traced by examining the cross sections of the samples from the wounded stem (*Figures 3–4*) In both kinds of the wounded stem, the first line of secondary laticifer produced upon wounding was visible next to the cambial cells around the wound 6 days after cutting Laticifer formation lasted for a longer period in the wounded stem without cover than in the wounded stem with cover and consequently produced more laticifers (*Figure 3*)

Effect of Lipoxygenase Inhibitors on Laticifer Differentiation Induced by Wounding.

The effects of DIECA and NDGA on laticifer differentiation induced by wounding were studied DIECA and NDGA are inhibitors of lipoxygenase, the key enzyme for JA biosynthesis as shown by Vick and Zimmerman[16] and Farmer *et al* [17] The lanolin containing one of the chemicals in varied concentrations was applied to the wound on the stem of epicormic shoot immediately after the stem was wounded by breaking epidermal cells In comparison with the treatment with lanolin containing no inhibitor, the treatments with the chemicals led to induction of fewer secondary laticifers (*Figure 5*)

JA or LA: Effect on Laticifer Differentiation Induced by Wounding

JA and LA were tested for their effect on laticifer differentiation induced by wounding The lanolin paste containing 0 07% JA or 0 2% LA was applied to the wound made by breaking epidermal cells of the stem of epicormic shoot More secondary laticifer was produced in the treatment by scraping plus JA or LA in comparison to the treatment with scraping only (*Figure 6*)

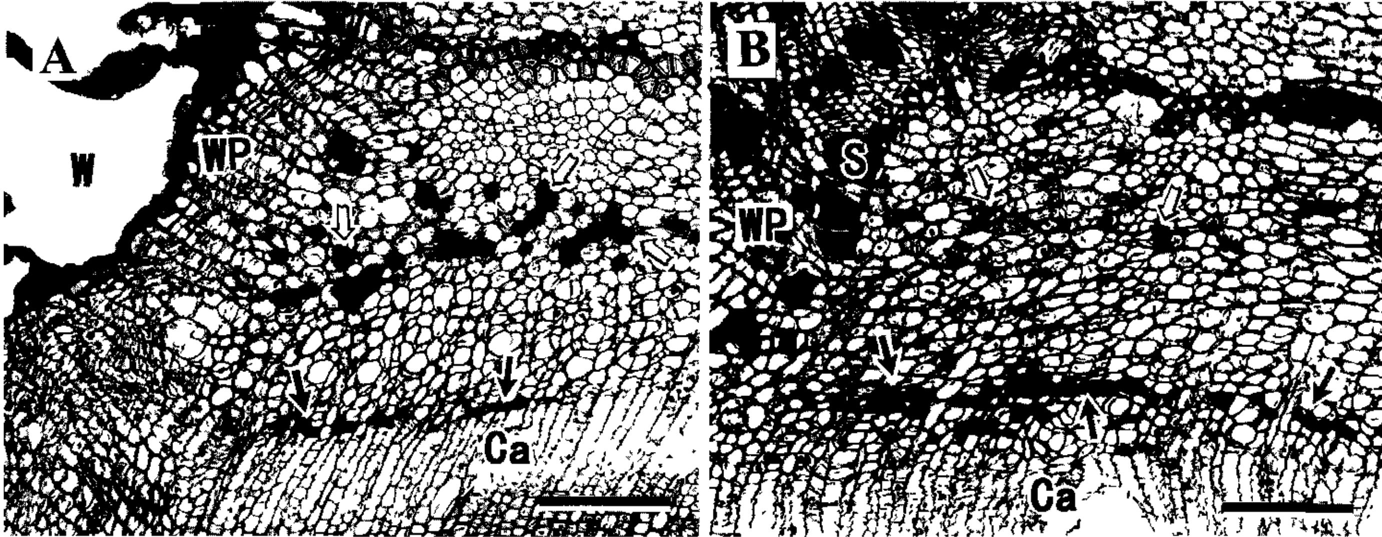

Figure 4 A–B. Cross-sections of bark, showing laticifer differentiation as influenced by cutting without covering: A, the first secondary laticifers (black arrows) induced 6 days after cutting; B, more secondary laticifers (black arrows) induced 30 days after cutting. White arrows, primary laticifers; Ca, cambium; S, sclereids; W, wound; WP, wound periderm. Bars = 50 μm.

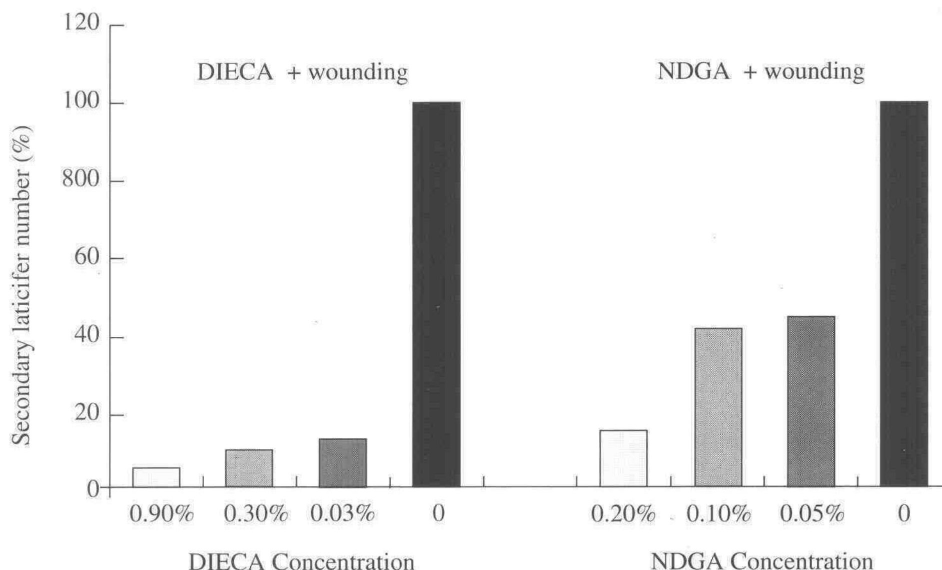

Figure 5. Effects of DIECA and NDGA on laticifer differentiation upon wounding by breaking epidermal cells, determined 40 days after treatment. (Results are means of five shoots.)

DISCUSSION

It has been demonstrated that laticifer differentiation in *Hevea* is inducible by mechanical wounding. The injury in varied degree inflicted by wounding in varied ways on the young stem of epicormic shoot led to the formation of secondary laticifer that did not occur in uninjured stem. Light injury such as by breaking the epidermal cells of the stem usually caused the formation of a line of secondary laticifer in the area roughly equal to that of the wound. Heavy injury such as by cutting the bark into the xylem of the stem resulted in the differentiation of more than one line of secondary laticifer in an area much larger than that of the wound. The first laticifer induced by wounding was visible as early as 6 days after wounding and at the same time as the formation of wound periderm (*Figure 4A*).

Available evidence indicates that the effect of wounding on the differentiation of laticifers in *Hevea* is mediated by JA. Detection in many plant species has shown that jasmonates accumulate in response to wounding (for reviews see Creelman and Mullet[12] and Seo *et al.*[9]) Application of JA results in the formation of laticifers in *Hevea*[10]. The laticifer differentiation in *Hevea* is induced by exogenously applied LA, the precursor of JA biosynthesis[14]. As shown in the present study, the wounding-induced laticifer differentiation in *Hevea* is enhanced by exogenous JA and hindered by lipoxygenase inhibitors, DIECA and NDGA. It is known that the lipoxygenase mediates an essential step in JA biosynthesis and the accumulation of JA in plants in response to wounding or the activation of the JA-induced genes can be blocked using inhibitors of lipoxygenase[17,18].

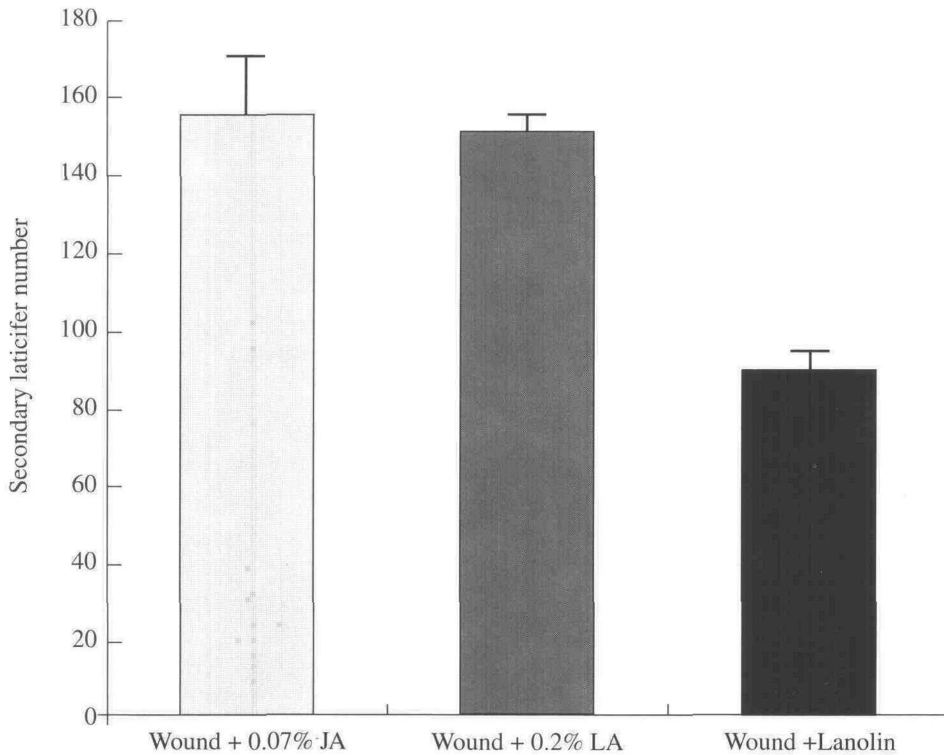

Figure 6. Secondary laticifer differentiation induced by wounding plus JA or LA, determined 40 days after treatment. (Results are means ± s.d. of five shoots.)

Based on the effect of JA on laticifer differentiation, some results from our experiments can be explained. More secondary laticifer induced by cutting might be attributed to more production of JA. Cutting not only made many cells broken but also caused latex to flow out of laticifer, and both cell injury and latex flow might induce the formation of JA. Laticifer in *Hevea* appear as a complex anastomosing system and latex in the laticiferous vessels is at a high turgor pressure, usually between 10 atm and 15 atm. When the laticifer is broken and the latex drained, the turgor pressure falls along the laticifer system in a considerable area of *Hevea* bark[19]. The change of the turgor pressure might trigger JA biosynthesis in or around the laticifers as suggested by Creelman and Mullet[20], and rapid induction of JA resulted from turgor loss caused by water deficit in soybean leaf. Latex flow therefore might cause JA biosynthesis, and consequently induce laticifer differentiation. The effect of JA could also account for the results of the experiment where in comparison with the wounded stem covered with lanolin paste and polyethylene membrane, the wounded stem without coverings had much more laticifers induced. From the uncovered wound, surface water loss must occur and result in water deficit of the stem tissue. This should induce JA biosynthesis as observed in *Arabidopsis* by Creelman and Mullet[20], and the

increased JA production might have led to the formation of more laticifers in the wounded stem without coverings.

As mentioned earlier, mechanical wounding can induce a series of changes in development of the wounded plant. These changes may be included in two categories: regeneration of lost cells, tissues or organs; and formation of defence structures. It is well established that JA plays an important role in the formation of defence chemicals when the plant is injured by insect, pathogens or physical factors *e.g.* mechanical wounding. JA is therefore expected to be involved in the formation of defence structures in response to mechanical wounding as well as biotic injury. As the present study shows, this might be true of *Hevea* laticifer because as a defence structure, the laticifer differentiated in response to wounding and this might be mediated by JA. We propose that JA is also a regulator for differentiation upon abiotic or biotic injury of other defence structures such as gum ducts or resin ducts in some plant species.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation of China (No. 39860013).

Date of receipt: September 2001
Date of acceptance: March 2002

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