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Regulation of Root Growth in *Lactuca sativa* L. Seedlings by the *Ent*-Kaurane Diterpenoid Epinodosin

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Abstract Epinodosin, an *ent*-kaurane diterpenoid isolated from *Isodon japonica* var. *galaucocalyx*, had a biphasic, dose-dependent effect on root growth and a strong inhibitory effect on root hair development in *Lactuca sativa* L. seedlings. Lower levels of epinodosin (25–100 μM) significantly promoted root growth, but higher concentrations (150–200 μM), by contrast, had inhibitory effects. In addition, all of the tested concentrations (20–80 μM) inhibited root hair development of lettuce seedlings in a dose-dependent manner. Further investigations on the underlying mechanism revealed that the promotion effect of epinodosin (25–100 μM) resulted from increasing the cell length in the mature region and enhancing the mitotic activity of meristematic cells in lettuce seedling root tips. On the other hand, epinodosin at higher concentrations inhibited root growth by strongly affecting both the cell length in the mature region and the division of meristematic cells. Comet assay analysis demonstrated that the decrease of mitotic activity of root meristematic cells was due to DNA damage induced by higher levels of epinodosin.

Keywords *Ent*-kaurane diterpenoid · *Lactuca sativa* L. · Root growth · Mitotic index · DNA damage · Root hair

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Introduction

To ensure the survival in the ecosystem, plants produce secondary metabolites with original chemical or biological features. These compounds can be introduced into the environment and interfere with the development of other vegetal organisms (D'Abrosca and others 2005). Terpenes, as the largest group of secondary metabolites in almost all plants, are involved in multiple ecological functions in plants, such as protection against herbivores and microbial diseases and attraction of pollinators, and in the phenomenon of allelopathy (Swain 1977; Fischer and others 1994; Calera and others 1995; Cangiano and others 2002; Macías and others 2002). Phytotoxic terpenoids and their possible involvement in allelopathy are covered in reviews on mono- and sesquiterpenes (Fischer 1986; Macías and others 2007). Many of these compounds are potent inhibitors of seed germination and the growth of several plant species (Duke and others 1988; Fischer 1991; Macías and others 2000a; Morimoto and Komai 2005; Cantrell and others 2007). In recent years, the role of diterpenoids in allelopathy has been supported (Macías and others 1999, 2000b). For example, Cangiano and others (2002) reported the effects of 20 *ent*-labdane diterpenes from *Ruppia maritima* and *Potamogeton natans* (aquatic plants) on aquatic organisms from different trophic levels. Morales-Flores and others (2007) reported that the natural diterpenes from *Croton ciliatoglanduliferus* Ort. inhibited photophosphorylation, electron transport, and the partial reactions of both photosystems in spinach thylakoids. In addition, Macías and others (2008) reported that helikauranoside A, a new *ent*-kaurane glycosylated diterpenoid isolated from the aerial parts of *Helianthus annuus* L., might be related to the allelopathic behavior shown by this species. It has been demonstrated that terpenoids could simultaneously display

stimulatory and inhibitory effects in seedling growth—that is, stimulate seedling growth at low concentrations and inhibit at high concentration (Asplund 1968; Bradow and Connick 1990; Fischer and others 1994; Jacob and others 2005). However, the large majority of the studies carried out on this phenomenon have referred to the phytotoxic effects when plant seedlings were exposed to various kinds of biotic and abiotic stress. Literature data on the mechanism for dual stimulatory and inhibitory effects on root growth are very scarce.

Isodon (Labiatae) is composed of approximately 150 species that are widely distributed in tropical regions of Africa and subtropical regions of Asia. *Ent*-kaurane diterpenoids are the main constituent in this genus and are especially abundant in fresh branches and leaves (Sun and others 2001). To date, more than 400 *ent*-kaurane diterpenoids have been isolated from these plants and many have displayed various biological activities such as antibacterial, anti-inflammatory, and antitumor activities (Sun and others 2001). Because they are perennial herbs, undershrubs, or subundershrubs, the aerial parts of *Isodon* plants wilt, fall to the ground, and are incorporated into the rhizosphere via degradative processes on an annual basis. However, whether these chemical components mediate interactions in the natural surroundings is still unknown.

Epinodosin, epinodosinol, rabdosin B, rabdosinate, lasiokaurin, and oridonin were isolated from branches and leaves of *Isodon japonica* var. *galaucocalyx* obtained from Gansu, China (Liu and others 2006). The structures were elucidated by IR, MS, and NMR. Our preliminary experiment indicated that epinodosin (a 6,7-seco-*ent*-kauranes enmein-type diterpenoid, Fig. 1) displayed both stimulatory and inhibitory effects on lettuce seedlings. A more detailed understanding of the mechanisms of these effects would be important as a first step in elucidating whether this diterpenoid might be capable of producing a natural ecological effect. Hence, epinodosin was selected to probe for possible modes of action in seedlings of *Lactuca sativa* L., which has been used extensively as a test organism because of its rapid germination and high sensitivity (Rasmussen and Einhellig 1979).

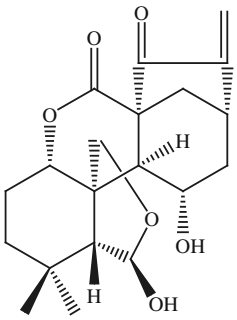

Fig. 1 Chemical structure of epinodosin, an *ent*-kaurane diterpenoid from the species of *Isodon*

Root growth depends on two basal developmental processes: cell division in the root apical meristem and elongation of cells that leave the root meristem (Scheres and others 2002). In our study, the elongation of cells in the mature region and the cell division of meristematic cells were both investigated to evaluate the effects of epinodosin on root growth in lettuce seedlings. In addition, the extent of DNA damage in root tip cells could be detected to elucidate the mechanism of the phytotoxic effects of epinodosin in lettuce seedlings by comet assay or single-cell gel electrophoresis (SCGE) assay, in which nuclei isolated from various tissues are exposed to electrophoresis (Navarrete and others 1997; Gichner and Plewa 1998; Koppen and Angelis 1998; Angelis and others 1999; Tice and others 2000). Root hairs, one of the smallest root structures and projections from root epidermal cells that increase the effective surface area available for nutrient and water uptake, can be used to evaluate the phytotoxic influence of natural compounds on root development.

The objectives of this study were (1) to determine the dual stimulatory and inhibitory effects of epinodosin in lettuce seedlings and (2) to probe the action modes of the diterpenoid.

Materials and Methods

Plant Materials and Chemicals

Lettuce (*Lactuca sativa* L.) seeds were obtained from the Agricultural Academy of Science, Gansu, China. Epinodosin was isolated previously in our laboratory from *Isodon japonica* (Burm. f) Hara var. *galaucocalyx* (maxin) Hara (Liu and others 2006). Ethidium bromide (EtBr) and normal-melting-point (NMP) and low-melting-point (LMP) agarose were purchased from AMRESCO Inc. (Solon, OH, USA). All other general laboratory reagents were obtained from Sango Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Culture Conditions and Treatments

Lettuce seeds were rinsed in tap water for 5 min, followed by three rinses in distilled water. Then seeds were placed into Petri dishes that contained two sheets of filter paper soaked with distilled water and allowed to germinate for 48 h at 20 ± 2°C and a photoperiod of 16:8 h light:dark. Twelve uniform 2-day-old seedlings were transferred to Petri dishes containing filter paper moistened with different concentrations of treatment solutions and incubated under the same conditions.

Epinodosin was dissolved in methanol and diluted to the desired concentrations with distilled water. The amount of

methanol was uniform for each treatment and never exceeded 1%, that is, below a level where root growth and root hair development were affected (data not shown).

Measurement of Net Growth Rates (NGR)

Seedlings were cultivated as described above. After germination, 12 seedlings per dish were incubated. Net growth rates (NGR) were measured to evaluate the influence of epinodosin on root growth according to the method of Pan and others (2001). The seminal root lengths of the seedlings in each treatment were measured before and after treatment. NGR was calculated as follows: (final length − initial length)/initial length. Three parallel dishes were examined per treatment and the experiment was repeated three times.

Measurement of Cell Sizes

To determine the influence of epinodosin on the cell size of lettuce seedlings, root tips (8 mm long) were excised from 2-day-old seedlings after treatment, fixed in an ethanol/acetic acid (9:1) solution overnight, and then washed in 90, 70, 50, and 30% ethanol sequentially. Root tips were then cleared with a chloral hydrate/glycerol/water solution (8:1:2 w/v/v) as described by Yadegari and others (1994). Cells were observed under an optical microscope (Nikon Eclipse E 400) equipped with an ocular micrometer. Cells at the root-hair-forming region of the roots were chosen to determine the matured cell size. The length of 20 mature cells from each root was measured, with six roots used per treatment. Three independent experiments were carried out, yielding the same statistically significant results.

Mitotic Activity

Seedlings were cultivated as described above. Squash techniques were modified from Akinboro and Bakare (2007). Primary roots of lettuce seedlings after treatment with epinodosin were fixed in freshly prepared ethanol–acetic acid (3:1 v/v) for 24 h, then transferred into 70% ethanol and stored at 4°C until use. The roots were hydrolyzed for 8–10 min with 1 M HCl at 60°C and then rinsed in distilled water (3–4 times). Root tips (1 mm) were excised and stained with carbol fuchsin for 10–15 min and the meristems were squashed under a coverslip to separate their cells. Four slides were prepared for each treatment, and at least 1000 cells were randomly examined under a Nikon Eclipse E400 optical microscope to determine the mitotic index, which was calculated as the number of dividing cells per 1000 observed cells. Three independent experiments were done, yielding the same statistically significant results.

Comet Assay

Isolation of nuclei from root tips and comet assay were performed following the method described by Gichner and others (2004, 2008) with some modifications. After incubation for 48 h, 80 seedlings of each treatment were used for the nuclei isolation and comet assay. The apical 2 ± 0.5-mm-long root tip of each seedling was removed and placed in a 7.5-cm Petri dish kept on ice and spread with 250 µl of cold 400 mM Tris buffer (pH 7.5). Using a fresh razor blade, the roots were gently sliced. The plate was kept tilted in the ice so that the isolated root nuclei would be collected in the buffer. Immediately, the obtained suspension was filtered twice through 30-µm-mesh stainless-steel cell filters, and the filtrate (200 µl) was collected in Eppendorf tubes. All operations were conducted under dim or yellow light.

Frosted microscope slides were coated with 0.5% normal-melting-point (NMP) agarose prepared with water at 50°C, dried overnight at room temperature, and kept dry in slide boxes until use. The nuclear suspension (50 µl) and 1% low-melting-point (LMP) agarose (50 µl) prepared with phosphate-buffered saline were added onto each slide at 40°C. The nuclei and the LMP agarose were gently mixed by repeated pipetting using a cut micropipette tip, and a coverslip was placed on the mixture. The slides were kept at 4°C for 15 min for the gel agarose to solidify. After removing the cover glass, the slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH > 13). The nuclei were incubated for 15 min to allow the DNA to unwind prior to electrophoresis at 0.74 V cm^{-1} (25 V, 300 mA) for 25 min at 4°C. After electrophoresis, the slides were rinsed three times with 400 mM Tris buffer (pH 7.5), stained with 80 µl EtBr (20 µg ml^{-1}) for 5 min, dipped in ice-cold water to remove the excess stain, and covered with a coverslip. The nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm.

Digital images were captured and analyzed using the CASP image analysis program (Końca and others 2003). Twenty-five randomly chosen nuclei were analyzed for each slide. Three slides were evaluated per treatment and each treatment was repeated three times. From the repeated experiments, the averaged median percentage of tail DNA (% tail DNA), tail length, and Olive tail moment [OTM, the product of the distance between the gravity centers of the DNA head and the DNA tail ($LX_{Gravity}$) and % tail DNA ($Tail_{DNA}$), $OTM = LX_{Gravity} \times Tail_{DNA}$ (Liu and others 2004)] were used to measure DNA damage for each treatment group. In total, at least 230 nuclei were evaluated for each treatment group.

Measurements of Root Hairs

Root hairs were evaluated according to the method of Yang and others (2004) with slight modifications. Seedlings (10) were randomly selected from each treatment. A 1-cm root segment was excised from each seedling at 0.5 cm behind the root tip, fixed in 70% ethanol, placed onto microscope slides, and covered by a glass cover for microscopic observation. The length and number of root hairs present in certain regions (424-μm root segment) were measured (3 times) from the ends and the middle of root segments with an ocular micrometer. Measurements for each treatment were determined from the ten root segments and mean values were calculated. Three independent experiments were done, yielding the same statistically significant results.

Statistical Analysis

All data are presented as mean ± SD. Data were subjected to analysis of variance (ANOVA), with significant differences among means identified by LSD multiple range tests using SPSS v11.5 (SPSS, Inc., Chicago, IL). Differences were considered significant at $p < 0.05$. To verify the relationship between pairs of data (root NGR/cell length; root NGR/mitotic index; mitotic index/prophase index) at the same epinodosin concentrations, linear regression analysis was performed and the coefficient of regression (r) and its level of significance (p) was determined.

Results

Effects of Epinodosin on Seedling Growth

The use of pregerminated seedlings in growth bioassays was a great advance in the effort to detect effects on growth processes avoiding previous resistance (Sanchez-Moreiras and others 2008). The roots of lettuce seedlings responded to epinodosin concentrations in a dual stimulatory and inhibitory way (Fig. 2a). Lower levels of epinodosin (25–100 μM) significantly promoted root growth and the root NGR reached a maximum at 50 μM. Inhibitory effects were observed at higher concentrations of epinodosin (150–200 μM) and the values of NGR significantly decreased in a dose-dependent manner (Fig. 2b).

Effects of Epinodosin on Elongation of Root Cells

To evaluate the influence of epinodosin on cell elongation, we measured the size (length and width) of matured cells at the root-hair-forming regions of lettuce roots (Fig. 3). Figure 4 shows that the length and the width of matured cells were both affected significantly. Epinodosin affected

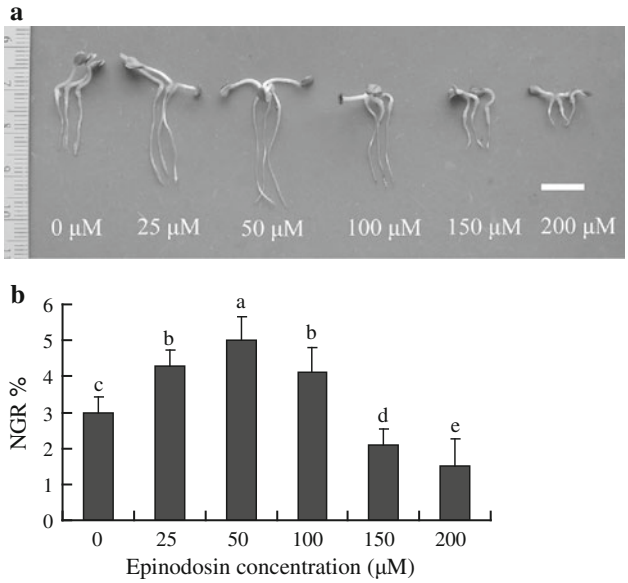

Fig. 2 Effect of epinodosin on root growth in lettuce seedlings. **a** Effects of increasing concentrations of epinodosin on the root growth of lettuce seedlings 48 h after treatment. **b** Root NGR in lettuce seedlings calculated after 48 h of treatment. *Different letters* indicate significant differences at $p < 0.05$. *Vertical bars* indicate SD. Scale bar = 1 cm

the cell length in a pattern that was similar to the NGR. Regression analysis revealed a positive correlation between NGR and cell length ($r = 0.993$, $p < 0.001$). Lower levels of epinodosin (25–100 μM) significantly increased cell length but decreased cell width, except at 100 μM. In contrast, after treatment with higher concentrations of epinodosin (150–200 μM), the cell length was reduced but cell width was increased significantly (Fig. 4).

Effects of Epinodosin on Mitotic Activity

To determine whether the root growth of lettuce seedlings treated with epinodosin involved changes in cell division in the root apical meristem, we examined the mitotic activity by squash techniques. Figure 5 showed the different phases of mitosis, which were calculated to determine the mitotic activity. Epinodosin affected the mitotic index in a pattern that was similar to the NGR ($r = 0.997$, $p < 0.001$). The mitotic index for root tip cells treated with lower levels of epinodosin (25–100 μM) significantly increased compared with controls, but at higher concentrations (150–200 μM), the mitotic index decreased (Fig. 6a). In addition, epinodosin affected the relative proportion of the various stages of mitosis in meristematic cells, with different stages showing different sensitivities to epinodosin (Fig. 6b). The number of meristematic cells in prophase was analogous to the mitotic index ($r = 0.938$, $p = 0.006$). However, the percentage of other phases did not change significantly compared with controls.

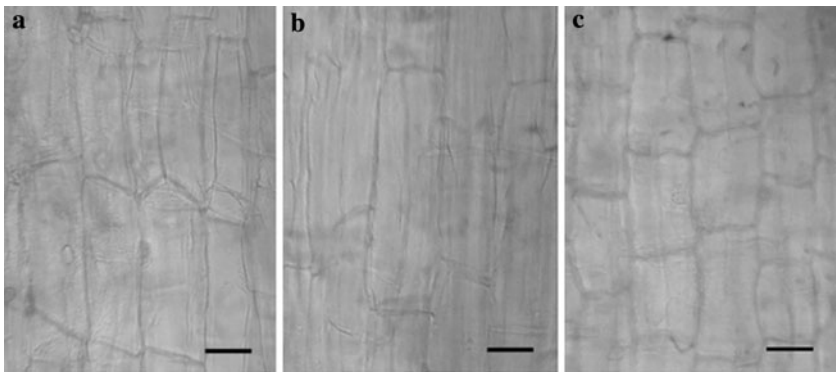

Fig. 3 Representative images showing the effects of epinodosin on the size of matured cells in lettuce roots 48 h after treatment. **a** Control. **b**, **c** Treatments with 50 and 200 μM epinodosin, respectively. Scale bar = 20 μm

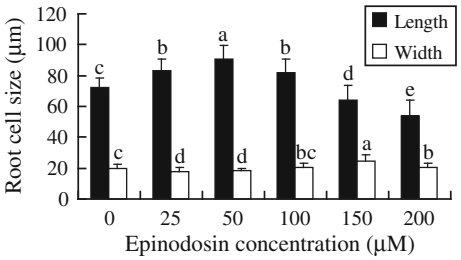

Fig. 4 Effect of epinodosin on the size of matured cells (length and width) in lettuce roots 48 h after treatment. Each datum represents average value from 120 cells. *Different letters* indicate significant differences at $p < 0.05$. *Scale bars* indicate SD

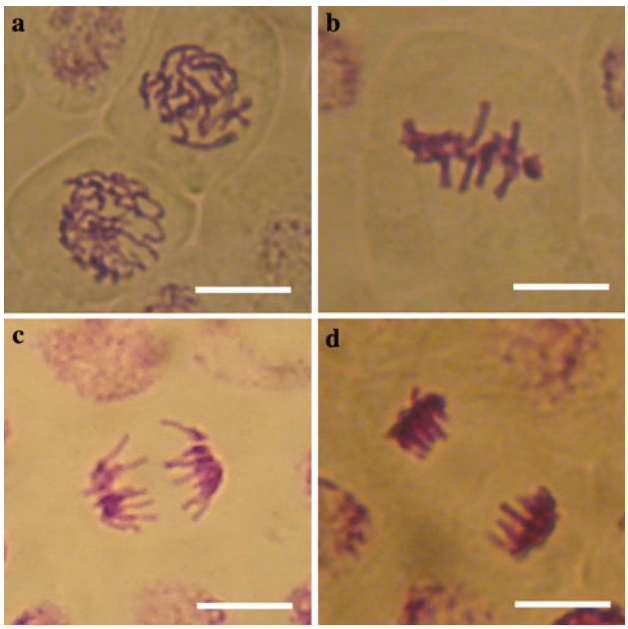

Fig. 5 Different phases of mitosis. **a** Prophase. **b** Metaphase. **c** Anaphase. **d** Telophase. Scale bar = 10 μm

DNA Damage in Root Nuclei Using Comet Assay

To determine whether the change in mitotic activity of lettuce root apical meristem cells involved DNA damage, DNA strand breaks were assessed by comet assay (Fig. 7).

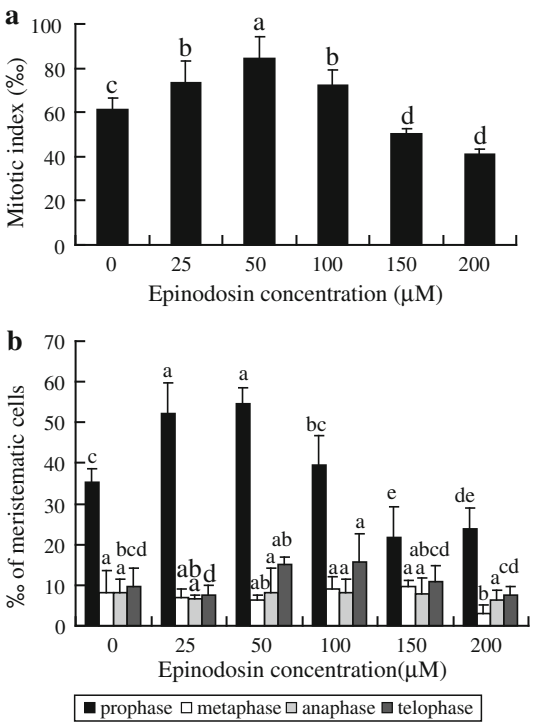

Fig. 6 Effect of epinodosin on the mitotic activity of lettuce seedling root apical meristem cells after treatment (48 h). **a** The mitotic index of root apical cells. **b** Percentage of different mitotic phases calculated relative to the total number of meristematic cells. *Different letters* indicate significant differences at $p < 0.05$. *Scale bars* indicate SD

Figure 8 shows a dose-dependent increase in DNA damage in the form of % tail DNA, tail length, and averaged median Olive tail moment (OTM) values by 150–200 μM epinodosin.

After treatment with epinodosin (150–200 μM) for 48 h, comet assay data clearly demonstrated a significant increase in average median % tail DNA values from 63.8 ± 10.2% to 78.6 ± 6.6%; in tail length values from 51.5 ± 8.1 pixels to 62.1 ± 10.4 pixels; and also in average median OTM values from 20.1 ± 4.8 pixels to 27.3 ± 5.3 pixels (Fig. 8). There was no significant difference in tail length or

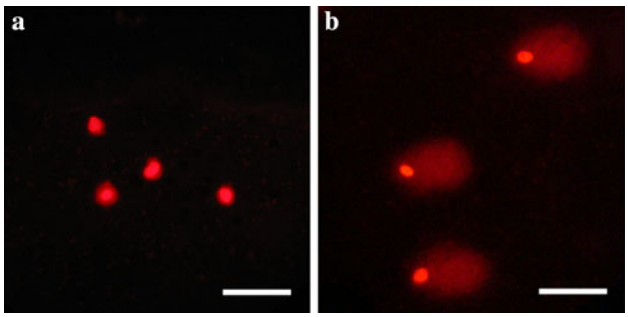

Fig. 7 Comet character showing tails of different lengths induced by epinodosin in nuclei of lettuce root cells for a period of 48 h in order to determine the dose–response for DNA damage: control (**a**) and treated with epinodosin 200 μM (**b**). Scale bar = 50 μm

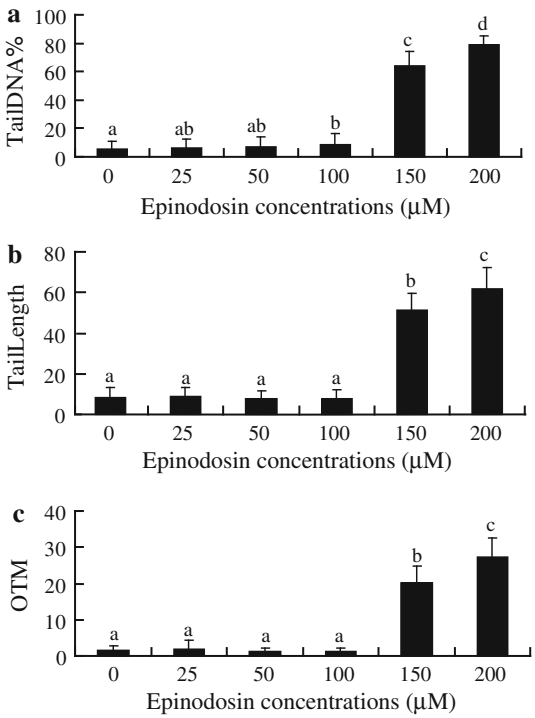

Fig. 8 Effect of epinodosin on the DNA damage of lettuce roots after treatment for 48 h. **a** The average median % tail DNA. **b** The average median tail length. **c** The average median Olive tail moment (OTM). *Different letters* indicate significant differences at $p < 0.05$. *Scale bars* indicate SD

OTM at the lower levels of epinodosin (25–100 μM). Only at 100 μM epinodosin was % tail DNA higher than that of the control, but the value was very low at only 8.9% (Fig. 8).

Effects of Epinodosin on Root Hair Development

Figure 9 shows that root hair development in lettuce seedlings was significantly inhibited by epinodosin after treatment for 48 h. The diterpenoid decreased the average

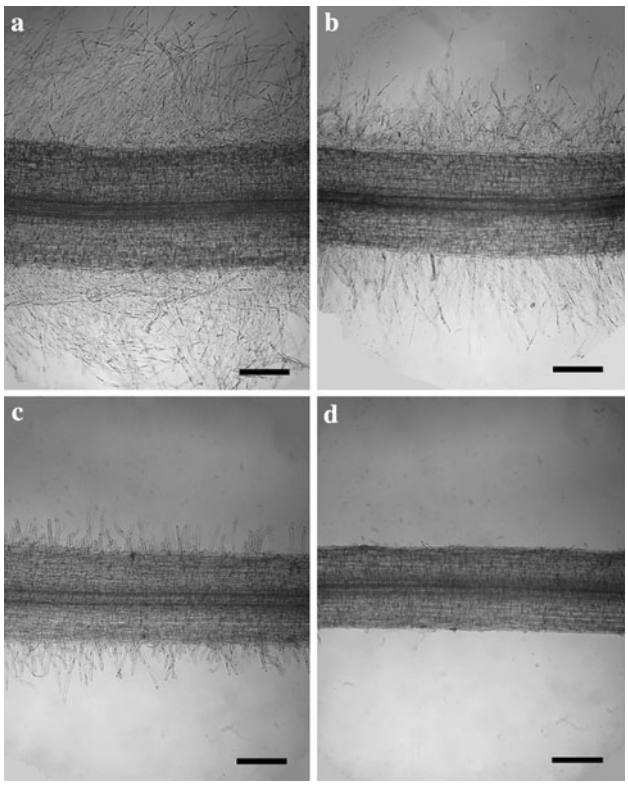

Fig. 9 Light micrographs of lettuce roots showing the inhibitory effects of epinodosin on root hair development 48 h after treatment. **a** Control. **b–d** Treatments with 20, 40, and 80 μM epinodosin, respectively. Scale bar = 200 μm

root hair length and root hair density in a dose-dependent manner at concentrations between 20 and 80 μM, with a larger effect on root hair length than on root hair density (Fig. 10). At lower concentrations of epinodosin (20 μM), there was no significant difference in the root hair density compared with controls (Fig. 10). When epinodosin concentrations were increased to 80 μM, only a few bulges were observed on root surfaces (Fig. 9d).

Discussion

In this article we have addressed the effect of epinodosin on root growth in lettuce seedlings. Our results indicate that epinodosin displays a dual stimulatory and inhibitory effect on lettuce root growth, that is, it stimulates seedling growth at low concentrations (25–100 μM) and inhibits growth at high concentrations (150–200 μM) (Fig. 2). Fischer and others (1994) reported that the mixture of terpenes could inhibit germination and root growth, but the pure substance promoted seedling growth at low concentrations. Kong and others (2002) found that the allelochemicals excreted by the roots of *Arachis hypogaea* before the four-leaf stage stimulated seedling growth of

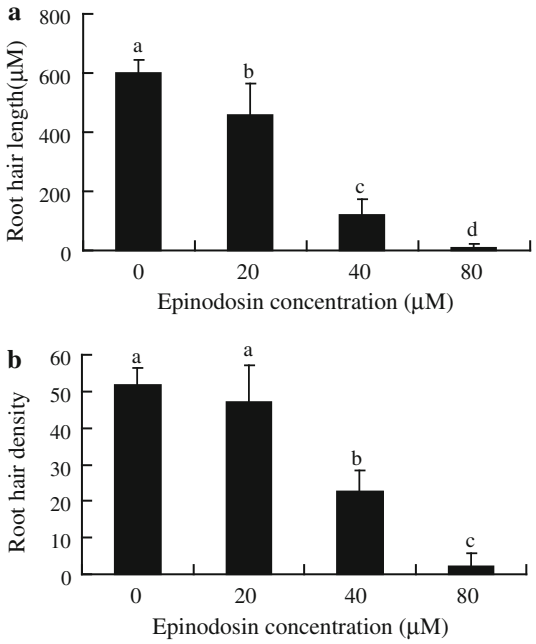

Fig. 10 Influence of epinodosin on root hair development in lettuce seedlings 48 h after treatment. Root hair development was evaluated by average root hair length (**a**) and root hair density (**b**). Root hair density was measured as the number of root hairs per 424-μm root segment. *Different letters* indicate significant differences at $p < 0.05$. *Scale bars* indicate SD

rice in low concentrations but inhibited it at high concentrations. Belz (2008) reported that hormesis (the stimulative effect of a toxin at low doses) might occur in a natural setting if doses released were low and it should be regarded as a potential low-dose component of plant/plant interference. Considerable research will be needed to understand the ecological conditions necessary for hormesis and the potential impact of low-dose stimulation by the diterpenoid.

Root growth arises from the proliferation of meristematic cells followed by cell expansion that results in root elongation. From our research it could be concluded that the inhibition of root growth induced by epinodosin at 150–200 μM was strongly correlated with both the changes of cell size at the mature region and the decrease in the mitotic index of root meristematic cells (Figs. 4, 6a). In addition, the number of meristematic cells in prophase followed a pattern similar to that of the mitotic index whereas the percentages of other mitotic phases did not change (Fig. 6b). Den Boer and Murray (2000) reported that upon induction of DNA damage or after alteration of intracellular redox homeostasis, the cell cycle slowed down at the G_1/S and G_2/M transition points. Therefore, we hypothesized that such a phenomenon depends on the general mechanism responsible for arresting the mitotic cycle in cells with damaged DNA (Fusconi and others 2007). To test this hypothesis directly, the comet assay was used to determine whether epinodosin could induce DNA damage of lettuce root tips. It is clearly seen in Fig. 8 that higher concentrations of epinodosin (150–200 μM) significantly induced a dose-dependent increase in DNA damage in the form of % tail DNA, tail length, and OTM values when compared with controls. These data were consistent with the supposition that DNA damage of lettuce root tips induced by epinodosin (150–200 μM) could lead to a decrease in mitotic activity. Cools and De Veylder (2008) reported that when DNA damage took place in meristems, plants would evolve DNA stress checkpoint mechanisms that arrested the cell cycle and activated the DNA repair machinery to preserve the genome content. Cell cycle arrest as a consequence of reduced DNA replication and the delay of the start of mitosis has been associated with an inhibition of the activity of cyclin-dependent kinases, cell cycle gene expression, and a concomitant activation of stress genes (Reichheld and others 1999; De Veylder and others 2007; Peres and others 2007).

Interestingly, the roots of lettuce seedlings responded to epinodosin concentrations in a biphasic way (Fig. 2). Concentrations of epinodosin lower than 100 μM had a significant stimulatory effect on root growth. To elucidate the mechanism of the promotion induced by the diterpenoid, we measured the size (length and width) of matured cells at the root-hair-forming region of lettuce roots. The results showed that lower levels of epinodosin (25–100 μM) significantly increased cell length but reduced cell width (Fig. 4). The data in Fig. 6 show that the mitotic index for root tip cells treated with lower levels of epinodosin (25–100 μM) significantly increased compared to the control, and an increase in the number of cells in prophase followed a pattern that was similar to the mitotic index (Fig. 6b). Thus, based on these data, we conclude that 25–100 μM epinodosin promoted root growth in lettuce seedlings by strongly affecting both the cell size and cell division. These findings are in good agreement with those in the literature, showing that radicle elongation was a result of cell division and/or cell elongation (Nicky and others 2007). The comet assay showed that after treatment with lower levels of epinodosin, there was a significant difference in % tail DNA values at 100 μM. It is possible that low levels of DNA damage induced by epinodosin at 100 μM could be repaired by lettuce seedlings and does not affect normal root growth. Further investigation is necessary to confirm the hypothesis.

In conclusion, our study demonstrated the dual stimulatory and inhibitory effects of epinodosin on root growth and phytotoxic effects on root hair development in lettuce seedlings. The underlying mechanism was that higher levels of epinodosin (150–200 μM) inhibited the root growth by strongly affecting both the cell length and cell division.

DNA damage induced by higher levels of epinodosin (150–200 μM) significantly correlated with a decrease of mitotic activity of root meristematic cells. The promotion of root growth resulted from enhanced cell length and increased mitotic activity. In addition, root hair development was significantly inhibited by epinodosin at all of the tested concentrations. Of course, further studies need to be carried out to confirm the role of epinodosin in mediating the ecological interactions in natural surroundings.

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