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Effects of Juglone on soybean root growth and induction of lignification

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ABSTRACT

We investigated the effects of application of juglone on the growth and cell viability, the activities of phenylalanine ammonia-lyase (PAL) and soluble and cell wall-bound peroxidase (POD), as well as the lignin content and its monomeric composition in the roots of soybean (*Glycine max* L. Merrill). Three-day-old seedlings were cultivated in half-strength Hoagland's solution (pH 6.0), with or without 5 µM juglone in a growth chamber (25°C, 12/12 h light/dark photoperiod, irradiance of 280 µmol m^{-2} s^{-1}) for 24 h. After juglone exposure, the root length and the fresh and dry weights decreased drastically, followed by major loss in cell viability. The soluble and cell wall-bound POD activities decreased, while PAL activity significantly increased just after juglone exposure. Thus juglone increased the lignin content and its *p*-hydroxyphenyl (H) monomer in the roots of soybean seedlings. These results suggested that the exogenous application of juglone allelochemical induced the inhibition of root growth in soybean due to excessive production of lignin.

Key words: Allelopathy, juglone, lignin, peroxidase, phenylalanine ammonia-lyase, soybean, roots.

INTRODUCTION

The plants are capable of releasing certain organic compounds into the environment, hence, the secondary metabolites may accumulate in the soil environment and thereby, influence the growth and development of plants growing in their vicinity, positively or negatively. This process is called allelopathy (15,33). Black walnut (*Juglans nigra* L.) is the oldest classical example of allelopathic plant (20). The plants which are sensitive to the presence of walnut in their adjoining landscape include tomato, potato, pea, pear, apple, cucumber, watermelon and bean. Besides some crops [alfalfa, wheat, corn, barley (2), soybean (16)] are also sensitive to the presence of black walnut. The roots, leaves and hulls of black walnut contain hydrojuglone which is oxidized to produce juglone (27) which is released into the soil (34). If it is not irreversibly absorbed or degraded, juglone may accumulate to levels that are sufficient to considerably influence the growth of other plants (32).

Application of juglone affects the plant growth at the seedling stage (34). Herein, its toxicity affects the normal functioning of vital cell processes (cell division, membrane transport, photosynthesis and respiration) (2). Recently, Babula *et al.* (1) demonstrated that

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the toxic effect of juglone operates by generating reactive species oxygen (ROS), which causes programmed cell death in the suspension culture of tobacco BY-2 species. However, in soybean, the application of juglone exhibits significant inhibitory effects on the photosynthesis, transpiration, stomatal conductance and respiratory functions of leaf and root and the relative growth rates of shoot and root (16). Further, it disrupts the normal functioning of electron transport system in the isolated mitochondria and chloroplasts (13) and reduces the rate of the H^+-ATPase activity in the root microsomal fraction (14).

Root growth is characterized by a high metabolic activity, hence, the roots are considered susceptible to the stress induced by allelochemicals. In an earlier study, Böhm *et al.* (4) verified that the reduction in the growth of soybean roots was associated with a premature occurrence of lignification in cell walls. This metabolic process that entails sealing a plant cell by the deposition of lignin is a necessary requisite for growth of all tissues of plants and hence, it is considered an important step during the process of root growth. Lignin is a complex component of cell wall, which comprises of phenolic heteropolymers that are covalently bound to both polysaccharides and proteins. It is mainly localized in the impermeable water transport conduits of the xylem and other supporting tissues (8). Lignin is synthesized by the phenylpropanoid pathway, which is also involved in the synthesis of a wide range of secondary products in plants such as, phenolic acids, flavonoids, tannins, coumarins (3,18). The first step in this pathway is the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) to produce cinnamate. The action of subsequent enzymes induces cinnamate to convert into different derivative metabolites, which are, further, converted to their corresponding monolignols. In the last step of pathway, peroxidase (POD) within the cell wall, in bound state, catalyzes the oxidative polymerization of the three *p*-hydroxycinnamil alcohols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol). These three alcohols give rise to three different units of the lignin polymer, *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively.

This study aimed (i). to test the hypothesis that exogenous application of juglone affects the phenylpropanoid pathway and eventually, increases the production of lignin, thus, reducing the root growth and (ii). to analyze the effects of juglone on the of PAL and POD activities and lignin content during the root growth of soybean seedlings.

MATERIALS AND METHODS

Soybean (*Glycine max* L. Merrill) seeds were surface-sterilized with 2% sodium hypochlorite for 2 min and rinsed extensively with deionized water and dark-germinated (at 25°C) on three sheets of moistened filter paper. Twenty-five 3-days-old seedlings of uniform size were mounted on an adjustable acrylic plate and then dipped into a glass container (measuring 10 x 16 cm) filled with 200 ml half-strength of Hoagland's solution (pH 6.0) with or without 5 µM juglone. This particular concentration was selected based on an earlier study, which reported the toxic effects of juglone on the roots of soybean (4). The container was kept in a growth chamber (25°C, light/dark photoperiod, irradiance of 280 $\mu mol\ m^{-2}\ s^{-1}$) for different time periods 6, 12, 18 and 24 h – of incubation. The roots were measured before incubation and at the end of experiments and the lengths were obtained by difference among them. The fresh root weight was determined immediately after incubation and the dry weight was estimated after oven-drying the samples at 80°C until it reached a constant weight. The results are the means ± S.E. of eight independent experiments. All the reagents used in this experiment were of the purest available grade or rather of the chromatographic grade.

Cell viability analysis

After 24 h of incubation, all seedlings were removed from the incubator to determine the loss of cell viability through Evans blue staining spectrophotometric assay (35). Additionally, all the freshly harvested roots were incubated for 15 min with 30 ml of 0.25% Evans blue solution. Further, the roots were washed in distilled water for 30 min to remove the excess and unbound dye. The excised root tips (3 cm) were soaked in 3 ml of *N*,*N*-dimethylformamide for 50 min at room temperature. The absorbance of the released Evans blue was measured at 600 nm, using deionized water as a blank. The loss of cell viability was expressed as the rate of absorbance at 600 nm of the treated roots in relation to untreated roots (control). The results are the means ± S.E. of five independent experiments.

Enzymatic assays

Phenylalanine ammonia-lyase (PAL) was extracted as described by Ferrarese *et al.* (11). The fresh roots (2 g) were ground at 4ºC in 0.1 M sodium borate buffer (pH 8.8). The homogenates were centrifuged (2200 *g*, 15 min) and the resultant supernatant was used as the enzyme preparation. The reaction mixture (100 µmoles sodium borate buffer, pH 8.7 and a suitable amount of enzyme extract in a final volume of 1.5 ml) was incubated (40°C, 5 min) for the PAL activity assay. In order to initiate the reaction, 15 µmoles of L-phenylalanine were added to the mixture. After 1 h the reaction was stopped by the addition of 50 µl of 5 N HCl. The resultant samples were filtered through a 0.45 µm disposable syringe filter and analyzed (20 µl) by means of a Shimadzu® Liquid Chromatograph. A reversed-phase Shimpack® CLC-ODS column (150 x 4.6 mm, 5 µm) was used at 30ºC. The mobile phase constituted the ratio methanol:water (70%:30%) with a flow rate of 0.5 ml min^{-1} for an isocratic run of 10 min. Absorption was measured at 275 nm. The product of PAL, *t*-cinnamate, was identified by comparing its retention time with standard values. PAL activity was expressed as µmol *t*-cinnamate h^{-1} g^{-1} fresh weight. The results are the means ± S.E. of four independent experiments.

For POD assay, fresh roots (0.5 g) were ground in a mortar with 0.01 g of polyvinylpolypirrolidone (PVP) and 5 ml of the 67 mM phosphate buffer (pH 7.0). The extract was centrifuged (2200 *g*, 5 min, 4°C) and the supernatant was used to determine the activity of soluble POD. The pellet was incubated in 1 M NaCl (2 ml, 1 h, 4°C). Further, the homogenate was centrifuged (2200 *g*, 5 min, 4°C) and the resultant supernatant contained the cell wall-(ionically)-bound POD. The enzymatic activities were determined according to the method given by dos Santos *et al.* (10). The reaction mixture (3 ml) contained 25 mM of sodium phosphate buffer (pH 6.8), 2.58 mM of guaiacol and 10 mM of H_2O_2. The reaction was initiated by adding the enzyme extract. The guaiacol oxidation was followed for 5 min at 470 nm and enzyme activity was calculated from the extinction coefficient (25.5 mM^{-1} cm^{-1}). POD activities were expressed as µmol tetraguaiacol min^{-1} g^{-1} fresh weight. The results are the means ± S.E. of six independent experiments.

Lignin quantification and monomeric composition

After the removal of other compounds by the phosphate buffer, Triton® X-100, NaCl and acetone, the lignin content of the root was determined from the protein-free cell wall fraction by the lignin-thioglycolic acid (LTGA) reaction (12). Lignin was expressed as mg LTGA g^{-1} dry weight. Further, the process of alkaline nitrobenzene oxidation was employed to determine the monomeric composition of lignin (35). The protein-free cell wall fraction (50 mg) obtained above was sealed in a Pyrex® ampule containing 1 ml of nitrobenzene and heated to 170°C for 90 min, all through this reaction the sample was occasionally shaken. Further, the sample was cooled at room temperature, washed twice with chloroform, acidified to attain a pH value of 2 with 2 M HCl and extracted twice through chloroform. The organic extracts were combined, dried, re-suspended in 1 ml of methanol and diluted in methanol/4% of aqueous acetic acid (20:80, v/v). All the evaluated samples were filtered through a 0.45-µm disposable syringe filter and analyzed by HPLC. The mobile phase constituted methanol/4% of aqueous acetic acid (20:80, v/v), with a flow rate of 1.2 ml min^{-1} for an isocratic run of 20 min. The quantification of the monomeric aldehyde (constituting *p*-hydroxybenzaldehyde, vanillin and syringaldehyde) products released by the nitrobenzene oxidation was performed at 290 nm by employing the corresponding standards. Results were expressed as µg monomer mg^{-1} cell wall. The results are the means ± S.E. of six independent experiments.

Statistical analysis: The experimental design was completely randomized and each plot was represented by a single glass container carrying 25 seedlings. The one-way variance analysis to test the significance of the observed differences was performed by Prism® package (Version 3.0, GraphPad Software Inc., USA). Means of exposure times were compared by the Tukey's test. Juglone-treated seedlings differing statistically from those of control were evaluated by the Student's *t*-test. $P \leq 0.05$ was adopted as the minimum criterion of significance.

RESULTS AND DISCUSSION

Root growth and cell viability

The data revealed that root growth (length, fresh and dry weights) increased in 5 µM juglone-treated and untreated (control) seedlings grown from 6 to 24 h (Table 1). However, the same data revealed that due to this substantial exposure to juglone, the length of the roots of the seedling decreased in value from 68.8% to 57.2% over the control. It was also recorded that the fresh weights of root significantly decreased from 24.3% to 15.7%, subsequent to an exposure of 6 to 18 h to juglone. Moreover, the dry weights of the roots also decreased from 13.3% to 26.1%. In addition, the toxicity of juglone caused visual differences among the exposed seedlings and the control group. After 24 h, the hydroponically grown seedlings appeared to be severely wilted, their roots were significantly stunted and more flexible and their apical meristems showed the appearance of a brown discolouration.

Table 1. Effects of juglone application on root length, root fresh and dry weights of soybean seedlings treated with or without 5 μM

		Exposure time			
Parameter	Treatment	6 h	12 h	18 h	24 h
Root length (cm)	Control	0.61 ± 0.016^{d}	1.51 ± 0.066^{c}	1.78 ± 0.042^{b}	2.97 ± 0.156^{a}
	Juglone	$0.19 \pm 0.001^{d*}$	$0.32 \pm 0.016^{c*}$	$0.59 \pm 0.018^{b*}$	$1.27 \pm 0.073^{a*}$
	%	68.8	78.8	66.8	57.2
Fresh weight (g)	Control	2.88 ± 0.059^{c}	2.73 ± 0.047^{c}	2.99 ± 0.135^{b}	3.06 ± 0.033^{a}
	Juglone	$2.18 \pm 0.037^{c*}$	$2.06 \pm 0.036^{d*}$	$2.52 \pm 0.060^{b*}$	$3.04 \pm 0.040^{a\,(ns)}$
	%	24.3	24.5	15.7	
Dry weight (g)	Control	0.15 ± 0.002^{d}	0.16 ± 0.001^{c}	0.18 ± 0.003^{b}	0.23 ± 0.006^{a}
	Juglone	$0.13 \pm 0.003^{c*}$	$0.13 \pm 0.004^{c*}$	$0.15 \pm 0.004^{b*}$	$0.17 \pm 0.003^{a*}$
	%	13.3	18.7	16.7	26.1

Mean values ± S.E. ($N = 8$) differing statistically (Tukey´s test) between exposure times ($P \leq 0.05$) are marked in letters. Mean values of juglone-treated seedlings differing statistically (Student´s *t*-test) from those of control ($P \leq 0.05$) are marked *. % represents inhibition of juglone in comparison to controls. (ns) = not significant.

The results also revealed a significant loss in the viability of cell caused by juglone over time than the corresponding controls (Fig. 1). Since, the uptake of Evans blue indicated that the loss of cell viability in the roots that were exposed to 5 μM of juglone were 3.7 – 9.1 times higher than the value observed for seedlings in the absence of the allelochemical. Several studies have demonstrated the inhibitory effects of the application of 1 μM to 1 mM of juglone on the growth of different plant species (17,21,26,27), including soybean (14,16,19). An increase in the uptake of Evans blue signalled the cell death, therefore, indicating that the roots of soybean are quite susceptible to the stress caused by juglone. Consistent with the cited reports, the data reported herein confirms the susceptibility of this plant species and reinforces the role of juglone as a strong allelochemical.

Lignification and related enzymes

The PAL activities increased in juglone-treated and untreated (control) seedlings grown for 6 to 24 h (Fig. 2). The roots that were exposed to allelochemical significantly increased the enzyme activity from 1.6 to 2.6-folds than controls. In general, soluble and cell-wall bound POD activities decreased in 5 μM juglone-treated and untreated (control) seedlings grown over time (Fig. 3). The presence of allelochemical reduced the soluble POD activities by 36% (at 6 h) and 24% (at 12 h) and cell wall-bound POD activities by 15% and 22% over the corresponding controls (Figs. 3A, B).

Whereas lignin content slightly decreased in untreated seedlings, its production substantially increased after juglone treatment (Fig. 4). As a consequence of this exposure, lignin content increased from 53% to 103% after 12 to 24 h, respect to corresponding controls (Fig. 4). The analysis of alkaline nitrobenzene oxidation products (Table 2) revealed that the content of the lignin monomer (*p*-hydroxyphenyl + guaiacyl + syringyl; H + G + S) increased by 10% in comparison to untreated roots. However, only the value of the H monomer increased significantly after being subjected to 5 μM of the juglone treatment, with respect to the control.

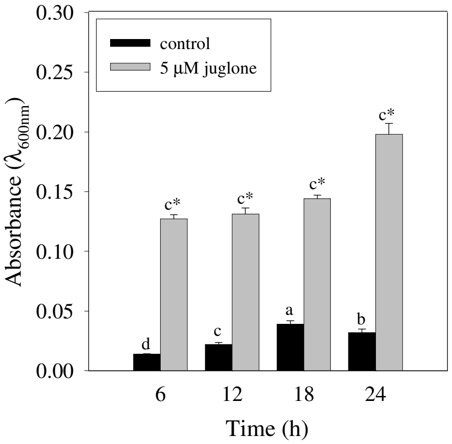

Figure 1. Loss of cell viability in the roots of the soybean seedlings treated with juglone. Mean values ± S.E. ($N = 5$) differing statistically (Tukey´s test) between exposure times ($P \leq 0.05$) are marked in letters. Mean values of juglone-treated seedlings differing statistically (Student´s *t*-test) from those of control ($P \leq 0.05$) are marked *.

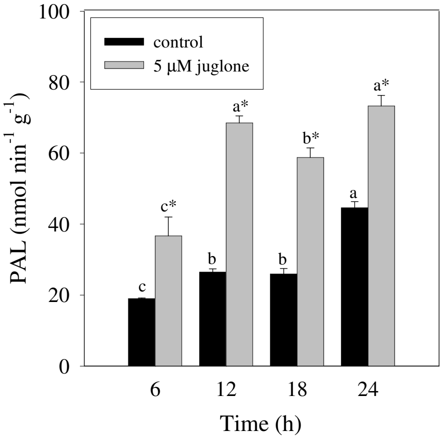

Figure 2. Effects of juglone on phenylalanine ammonia-lyase (PAL) activities. Mean values ± S.E. ($N = 4$) differing statistically (Tukey´s test) between exposure times ($P \leq 0.05$) are marked in letters. Mean values of juglone-treated seedlings differing statistically (Student´s *t*-test) from those of control ($P \leq 0.05$) are marked *.

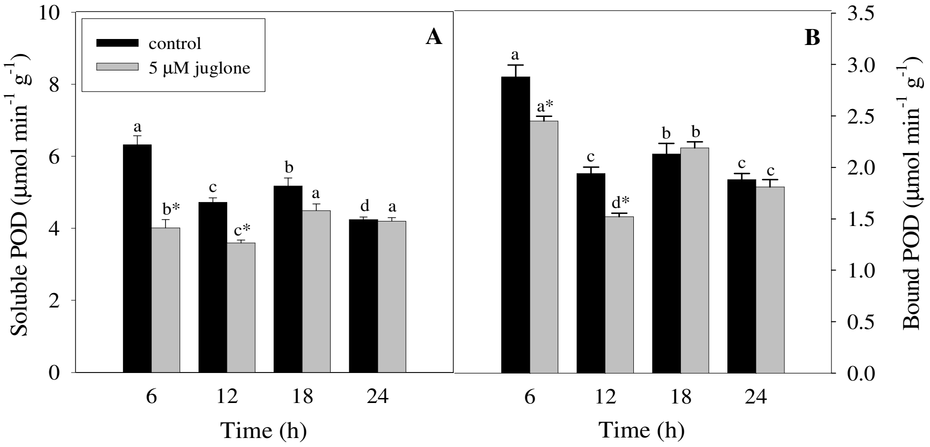

Figure 3. Effects of juglone on soluble (A) and cell wall-bound (B) peroxidases (POD) activities. Mean values ± S.E. ($N = 6$) differing statistically (Tukey´s test) between exposure times ($P \leq 0.05$) are marked in letters. Mean values of juglone-treated seedlings differing statistically (Student´s *t*-test) from those of control ($P \leq 0.05$) are marked *.

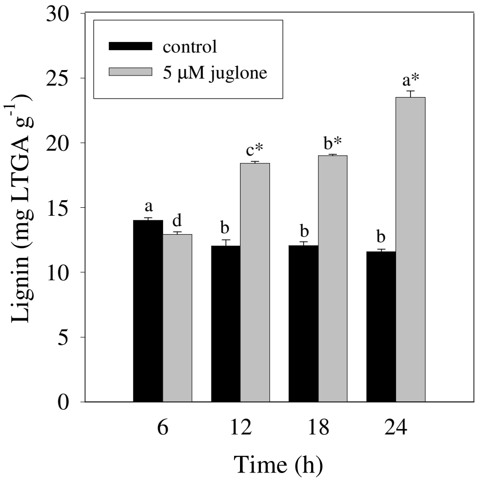

Figure 4. Effects of juglone on lignin contents. Mean values ± S.E. (N = 6) differing statistically (Tukey´s test) between exposure times ($P \leq 0.05$) are marked in letters. Mean values of juglone-treated seedlings differing statistically (Student´s t-test) from those of control ($P \leq 0.05$) are marked *.

Table 2. Effects of juglone application on lignin monomer composition of soybean roots untreated (Control) or treated with 5 µM juglone for 24 h. The results are expressed as µg monomer mg^{-1} cell wall.

Lignin monomer	Control	Juglone
H, *p*-hydroxyphenyl	0.16 ± 0.005	0.23 ± 0.016*
G, guaiacyl	1.74 ± 0.032	1.86 ± 0.048^{ns}
S, syringyl	0.23 ± 0.005	0.25 ± 0.009^{ns}

* Mean values ± S.E. (N = 5) differed significantly (Student´s t-test, $P \leq 0.05$) from those of controls. ns = not significant.

An important fact that was revealed in this investigation was that exposing the soybean seedlings to 5 µM of juglone inhibited their root growth and this change was followed by significant increases in the activities of PAL (Fig. 2) and lignin content (Fig. 4). It is known that most plants, especially in their early seedling growth, are highly susceptible to several soil allelochemicals (15,33); this may be attributed to the high rate of metabolic activity and the initiation of root lignification at this particular stage (8). These results suggest an evident induction of the lignification process since a link has been observed to be present between allelopathic stress and the production of root lignin in some plant species (6,10,25). As described earlier, the biosynthesis of lignin involves the polymerization of the monolignols that are primarily derived from the phenylpropanoid pathway, which commences with the deamination of phenylalanine by PAL to form cinnamate, followed by the other derivatives. By the action of subsequent enzymes, these phenylpropanoid derivatives are converted into corresponding monolignols, which are polymerized by POD in *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units into the lignin (3).

An increase in the activity of PAL is considered to be a response of plants to various biotic and abiotic stresses. The obtained results revealed that the exposure to the allelochemical prompted significant increases in the enzyme activities; this is yet another strong indicative of its toxic effect on the roots of soybean. The resultant evidence strengthens these results, since the addition of different allelochemicals also increased the PAL activities (9,10,25,28). On the contrary, an exposure to juglone significantly reduced the activities of the soluble and cell-wall bound POD in the roots of soybean (Figs. 3A and B). Some researchers have reported subsequent increases in the POD activity under the action of the allelochemical (7,23,24). It is well known that POD catalyzes the oxidation process of diverse phenolic substrates and is often regarded as an antioxidant enzyme that protects cells from the destructive influence of the oxygen radicals. Moreover, the cell wall-bound POD is involved in the process of lignification (22). However, these possibilities could not be inferred from the available data since the production of lignin (Fig. 4) was not accompanied by an increase in the rate of the POD activities (Fig. 3). These results are in accordance with those generated by the study previously reported by Böhm *et al.* (4). A possible explanation for this could be that the POD isozymes that are responsible for the cross-linking of the cell-wall were not suitably extracted from the roots, at least under the present experimental conditions. Another possible explanation can be that although the napthoquinones are cell permeable and readily undergo the redox reactions and, therefore, serve as the producers of intracellular superoxide (O_2^-) and H_2O_2 (29), yet these reactive oxygen species (ROS) might be scavenged by the cellular defensive system (for example, the superoxide dismutase and the catalase). As mentioned earlier, juglone is able to generate ROS leading to a programmed death of the cells of the tobacco BY-2 species (1). In spite of these evaluations and results, additional data is required to conclusively prove a link between the ROS causing cell death and the effects of juglone in soybean.

However, it is quite evident that the application of juglone inhibits the plant growth by reducing the rate of activities like photosynthesis and respiration as well as by increasing the level of oxidative stress (34). Irrespective of its correct mechanism of action, it is remarkable to note the characteristic of juglone to be able to increase the production of lignin (Fig. 4), hence, altering its monomeric composition (Table 2). In a previous study, Böhm *et al.* (4) observed that 5 μM of juglone clearly increased the lignin content in the roots of soybean. The cell walls become lignified due to a reduction of the cell expansion, either when the cell is under stress or when it differentiates to a particular specialization, notably, the xylem (5). The anatomical observations confirm these possibilities. A reduction in the size of the anatomical tissues, such as, the xylem vessel and the bundle radius of the stem of cucumber (30) and muskmelon (31) may be the probable cause of inhibition in plant growth as induced by the allelochemical. In the reports given by these authors (30, 31), the narrowing of the xylem vessels was considered as a probable defense mechanism of the seedlings to limit the juglone translocation, therefore, hindering the passage of water and nutrients from the roots to the leaves. In addition, Hejl and Koster (14) have shown through experiments that the application of juglone mediates its effects on soybean by disrupting the H^+-ATPase activity and by reducing the rate of uptake of water and oxygen by the roots. By consequence, the allelochemical may affect the metabolism of the root cells by disrupting the function of the root plasma membrane.

CONCLUSIONS

This study revealed that the application of juglone to soybean seedlings caused the following effects: (i). Decreased the root growth of soybean followed by a significant loss in the cell viability; (ii). Increased rate of PAL activity, which probably influenced the production of the lignin-related phenylpropanoid derivatives; and finally, (iii). Strongly increased the production of lignin thus altering its H monomer. Therefore, juglone plays major role in lignification of certain plants. The juglone-induced inhibition of root growth of soybean may be due to excessive production of lignin caused by the exogenously applied allelochemical.

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