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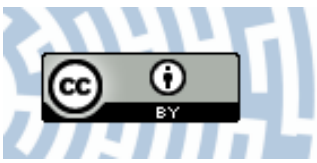

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MP: idea of the study; RG, EK, KS, PZR, AMH: microscopic analysis and photographs; EK, RG, AMH, KS: manuscript preparation

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ORIGINAL RESEARCH PAPER

Morpho-histological analysis of tomato (*Solanum lycopersicum* L.) plants after treatment with juglone

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Abstract
Juglone is a substance that limits plant growth and has a toxic effect on plant development. In this study, we analyzed the influence of juglone at two different concentrations (10^{-3} M and 10^{-4} M), which were applied to different parts of *Solanum lycopersicum* L. plants (root system, stem after decapitation, and surface of a younger leaf or after autografting) for a short period of time (7 days), on the morphology and histology of stems. At a lower concentration, juglone had positive effects on plant growth, which resulted in an increase in interfascicular cambial cell divisions, faster development of a continuous cambium layer along the stem circumference, and development of fibers. Additionally, under the influence of juglone, the number of developing leaves increased and adventitious roots developed. The results are discussed based on the current literature concerning the reaction of plants to juglone and to stress conditions.

Keywords
histology; cambium; fibers; juglone; morphology; tomato

Introduction

Juglone (5-hydroxy-1,4-naphthoquinone) is secreted by trees of the Juglandaceae family, especially by *Juglans regia* and *Juglans nigra* [1,2]. Chemically, juglone is a phenolic compound that belongs to the naphthoquinone class.

It has been repeatedly demonstrated that juglone has a toxic effect on plant growth. The most extensive studies have been conducted on corn and soybean [3]. It has been shown that juglone can inhibit the growth of shoots and roots and that it also interferes with photosynthesis and transpiration processes. Despite the large amount of published data concerning the biological activity of juglone, little is known about the mechanisms of its toxic effects on plant growth [2,4,5]. Studies on corn and soybean showed that juglone is responsible for the inhibition of plasma membrane H^+-ATPase, resulting in the inhibition of the uptake of water and nutrients by the roots (for review see [5]). The inhibition of root elongation by limiting the transport of protons from the cytoplasm to the apoplast was also reported [6]. This transport is regulated by the activity of the ATP-dependent proton pump that is located in the plasma membrane. In addition, juglone reduces the content of chlorophyll, interferes with the functioning of the mitochondria, and induces the formation of reactive oxygen species [5].

Many plants are sensitive to juglone. The most sensitive species include tomato, potato, pea, cucumber, apple, corn, soybean, azalea, and many others. There are several species that are not susceptible to juglone, e.g., onion, Jerusalem artichoke, sugar beet, and certain species of beans. The current literature also reports [7] a beneficial influence of juglone for some species. Research on one of the varieties of cotton showed that juglone at a 10^{-3} M concentration accelerates germination and increases growth [8].

Tomato belongs to the Solanaceae family. This vegetable species is widely used as an experimental plant in many areas of research and has significantly contributed to our understanding of the mechanisms underlying the influence of the environment on plant growth, development, and response to various external factors such as temperature [9], drought and salinity [10,11], or light conditions [12].

The present studies were initially undertaken to analyze the harmful effect of juglone on tomato plants, but when it appeared that a 10^{-4} M juglone concentration increased growth, it was decided to focus the study on the histology (plus certain morphological traits) of *Solanum lycopersicum* L. plants that were treated with juglone for a short period of time and to analyze the cellular events that occur during this process. The obtained results can contribute not only to increasing our basic knowledge of the influence of juglone on tomato growth, but can also be the starting point for applications studies.

Material and methods

Material

Seeds of *Solanum lycopersicum* L. 'Moneymaker' were germinated on Petri dishes with wet blotting paper for 6 or 7 days at 23 ±1°C in darkness. Seedlings were transferred to pots with a soil and vermiculite mixture (2:1 v/v) and grown at a temperature of 23 ±1°C, a relative humidity of 35% and a 16-h photoperiod with a light intensity of 40 μmol m^{-2} s^{-1} (cool white fluorescence lamp; OSRAM). Plants were watered every 2 days with Hoagland's solution [4]. For each experiment, 28-day-old plants were used.

Juglone treatment

Juglone (Sigma Aldrich) was dissolved in 5 mL of ethanol, made up to 1000 mL with distilled water (for experiments with juglone application in lanolin paste), or in Hogland's solution (for watering plants) in order to get a concentration of 10^{-3} M (lower concentrations were obtained by the dilution of the stock solution). The juglone treatment lasted for 7 days for each experiment. Six plants were used in each experiment and there were six replicates.

Healthy plants were treated with juglone in the following ways:

1. A juglone solution at a 10^{-3} M or 10^{-4} M concentration was used to water the plants; watering was repeated every 2 days during the 1-week period. Control plants were treated in the same way but without the addition of juglone.
2. A juglone solution at a 10^{-4} M concentration was mixed with lanolin at a 1:1 weight ratio and this mixture was applied on the stem after decapitation (the lanolin paste protected the cut surface against drying) or on the leaf surface after gentle abrasion of the cuticle with the use of polishing paste (lanolin protected against drying); the lanolin paste was replaced every 2 days. Control plants were treated in the same way but the lanolin was mixed with demineralized water.

Autografting procedure

28-day-old plants with healthy cotyledons and epicotyl length of about 1–1.5 cm were autografted according to the method described by Jeffree and Yeoman [13]. Mid-epicotyl was cut transversely with a razor blade and the scion was carefully placed back on the stock. Toothpicks and parafilm were used to support the grafted stems. Immediately after grafting, plants were treated with juglone as described above.

Morphology and histology

Observations of plant morphology were carried out using an Olympus (Japan) SZH10 stereomicroscope. For histological analysis, samples were fixed in FAA, dehydrated in an

ethanol series, and embedded in Steedman's wax [14,15]. Longitudinal or cross sections (7 μm thick) were cut with a rotary microtome HYRAX M40 (Zeiss, Germany) and collected on microscopic slides covered with Haupt's adhesive. Sections were stained with aqueous solutions of 0.05% Toluidine Blue O (Sigma) for 10 min, then rinsed three times with demineralized water. Observations and photography were carried out using a Nikon SMZ 1500 dissecting microscope or Nikon Eclipse Ni-U bright field microscope equipped with a Nikon Digital DS-Fi1-U3 camera with corresponding software (Nikon, Japan). Figures were edited in Corel Draw X5 (Corel Corp., Canada) and Paint.Ink (Microsoft Corp., USA).

Results

The appearance of control and treated plants

As was pointed out above, the plants treated with juglone at the lower concentration were in much better condition in comparison to control plants or plants treated with the higher juglone concentration (Fig. 1). The height of the control plants was ca. 25 cm, the height of those treated with the lower juglone concentration exceeded 30 cm, while that of plants treated with the higher juglone concentration was less than 20 cm. These values of the growth parameters are only illustrative, as statistical analysis was not performed because of the differences between individual plants.

Fig. 1 Representative appearance of control plants (**a**) and plants treated with juglone at 10^{-4} M (**b**) and 10^{-3} M (**c**). Scale bars: 5 cm.

Plants treated with the lower juglone concentration developed more leaves and their size was also larger in comparison to the control plants. Plants treated with juglone to the cut surface or to the leaf surface reacted similarly (data not shown).

Plants treated with juglone at 10^{-3} M were shorter and had fewer leaves compared to the control plants.

Stereomicroscopic observations of the stem surface of the juglone-treated plants revealed the presence of adventitious roots (AR) and this was a constant characteristic of plants regardless of the juglone concentration (Fig. 2). Also, the color of epidermal cells in the plants that were treated with juglone changed to purple (Fig. 2).

The next morphological difference between the plants treated with juglone and the control plants was the presence of trichomes that were visibly longer in the juglone-treated plants regardless of the concentration and method of juglone application (Fig. 2a–c).

Juglone applied to the graft union

Application of juglone accelerated the process of graft union formation, which was manifested by the faster fusion of the stock and scion in comparison to the control

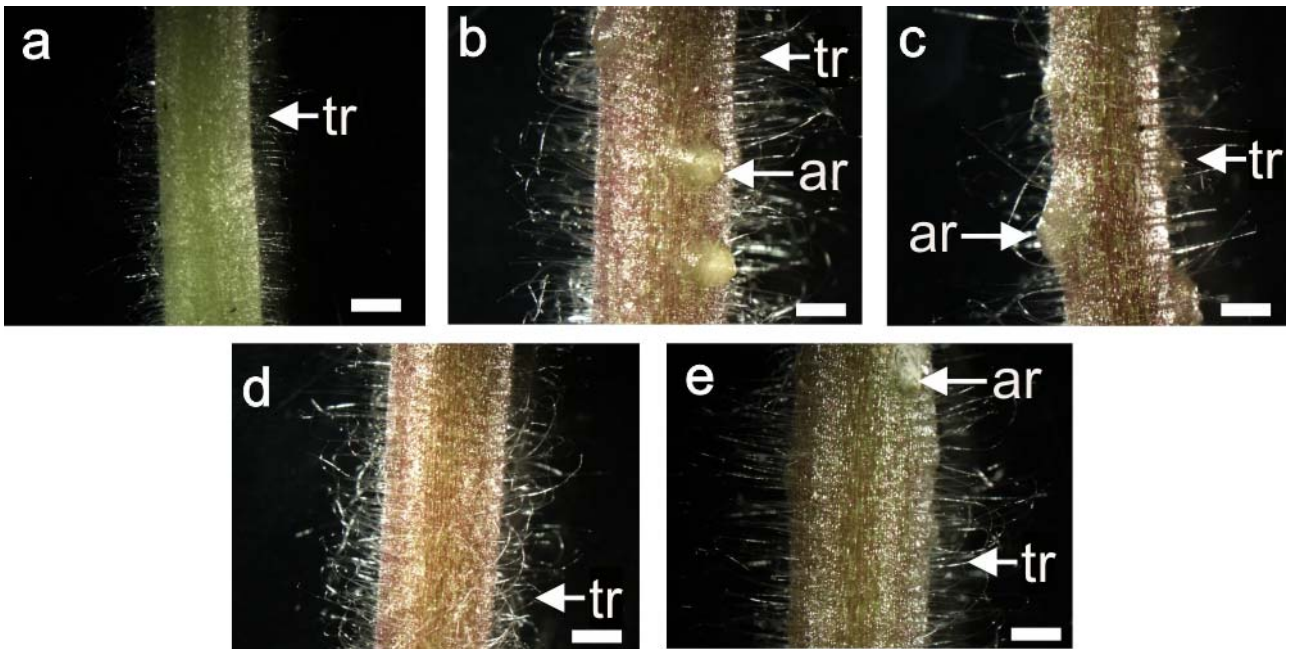

Fig. 2 Stem surface of a control plant (**a**); treated with juglone at a concentration of 10^{-4} M applied to the root system (**b**); treated with juglone at a concentration of 10^{-3} M applied to the root system (**c**); treated with juglone applied to the cut surface after decapitation (**d**); treated with juglone applied to the leaf surface (**e**). tr – trichomes; ar – adventitious root. Scale bars: 1 cm.

plants (Fig. 3). The shape of the juglone-treated grafted stems was uniform, whereas in the control plants differences in diameter (caused by a more pronounced growth of the upper part of the graft) between scion and stock were observed. The graft union in the juglone-treated plants was completely filled with callus cells.

Histological analysis

Longitudinal and cross sections through the epicotyls of the control and juglone-treated plants showed differences in cambial cell divisions and the development of the cambium in the interfascicular area. All of the features mentioned above, as well as differentiation of fibers and the development of the adventitious roots, were more pronounced in the juglone-treated plants.

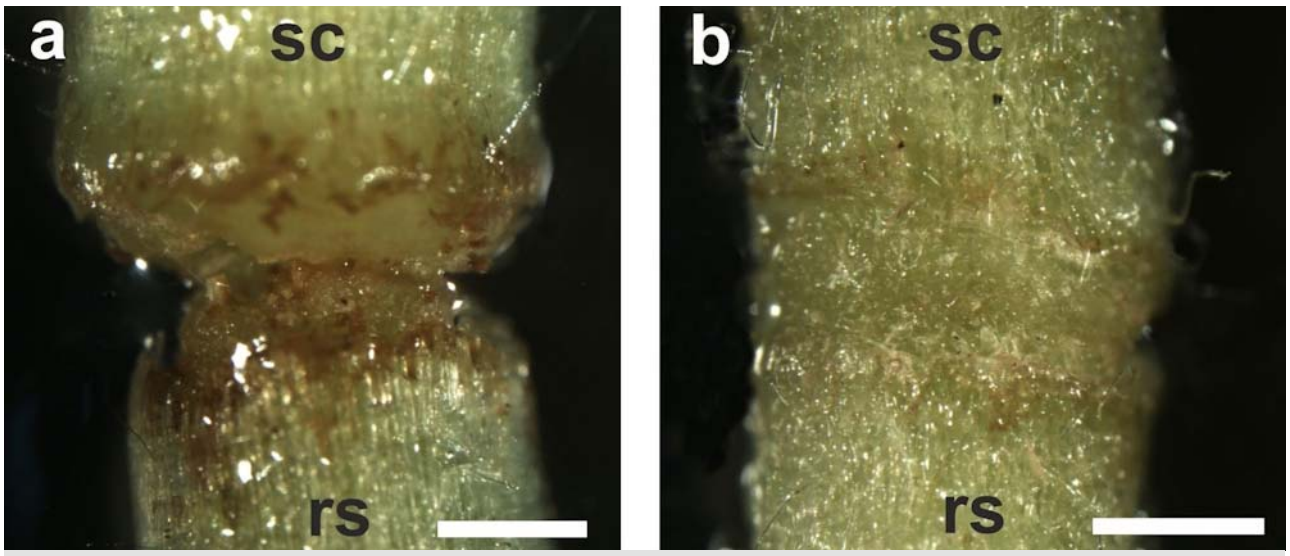

Fig. 3 Morphology of the graft union of a plant untreated with juglone (**a**) and a plant treated with juglone at a concentration of 10^{-4} M (**b**). sc – scion; rs – rootstock. Scale bars: 1 cm.

The histology of the control plants was typical. The central part was occupied by pith, the vascular bundles were distributed uniformly along the stem circumference, and the cambium was present mostly as a fascicular one (Fig. 4a). After juglone application to the root system, the cambium was uniformly present along the stem circumference (Fig. 4b). The number of cambial cells in radial rows was higher in comparison to the control plants. Both methods of stem treatment (juglone applied on the decapitated stem or on the leaf surface) resulted in similar changes in stem histology (Fig. 4c,d). Juglone application to the root system resulted in the abundant development of fibers (Fig. 4b). Because the number of daughter cells in radial files in the cambial zone and the number of cambial zone radial files along the circumference were higher in juglone-treated plants, it can be stated that cambium was more active in these plants in comparison to control plants.

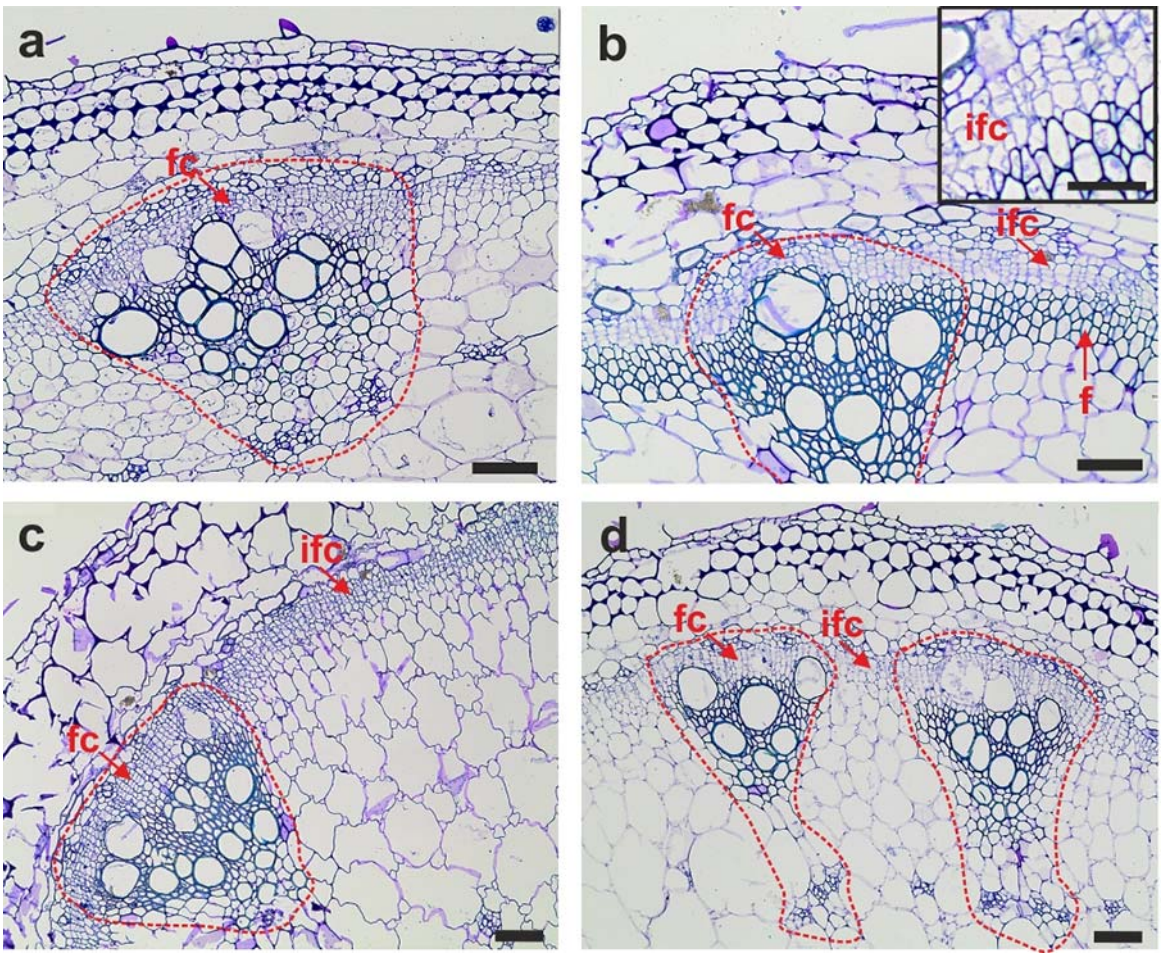

Fig. 4 Cross section of a control plant (**a**) and a juglone-treated plant at a 10^{-4} M concentration applied to the root system (**b**); applied to the cut surface after decapitation (**c**); applied to the leaf surface (**d**). Note the abundance of differentiated phloem fibers within the outer phloem/protophloem area as well as interfascicular xylem fibers in **b**. fc – fascicular cambium; ifc – interfascicular cambium; f – fibers; dotted line – vascular bundle. Scale bars: 100 μm.

The plants that were treated with juglone after autografting were characterized by faster connection of the scion and stock that was caused by the more abundant cell divisions as well as a faster dedifferentiation and/or differentiation compared to the untreated plants (Fig. 5a,b).

Juglone treatment resulted in faster union formation between the scion and stock, which was manifested in the filling of the gap formed after stem cutting with callus and the presence of dedifferentiated cells (Fig. 5a,b). The arrangements of cells, especially the tracheary elements, were disturbed in the area of the union and were manifested by the presence of cells whose longitudinal axis was perpendicular to the long axis of the stem.

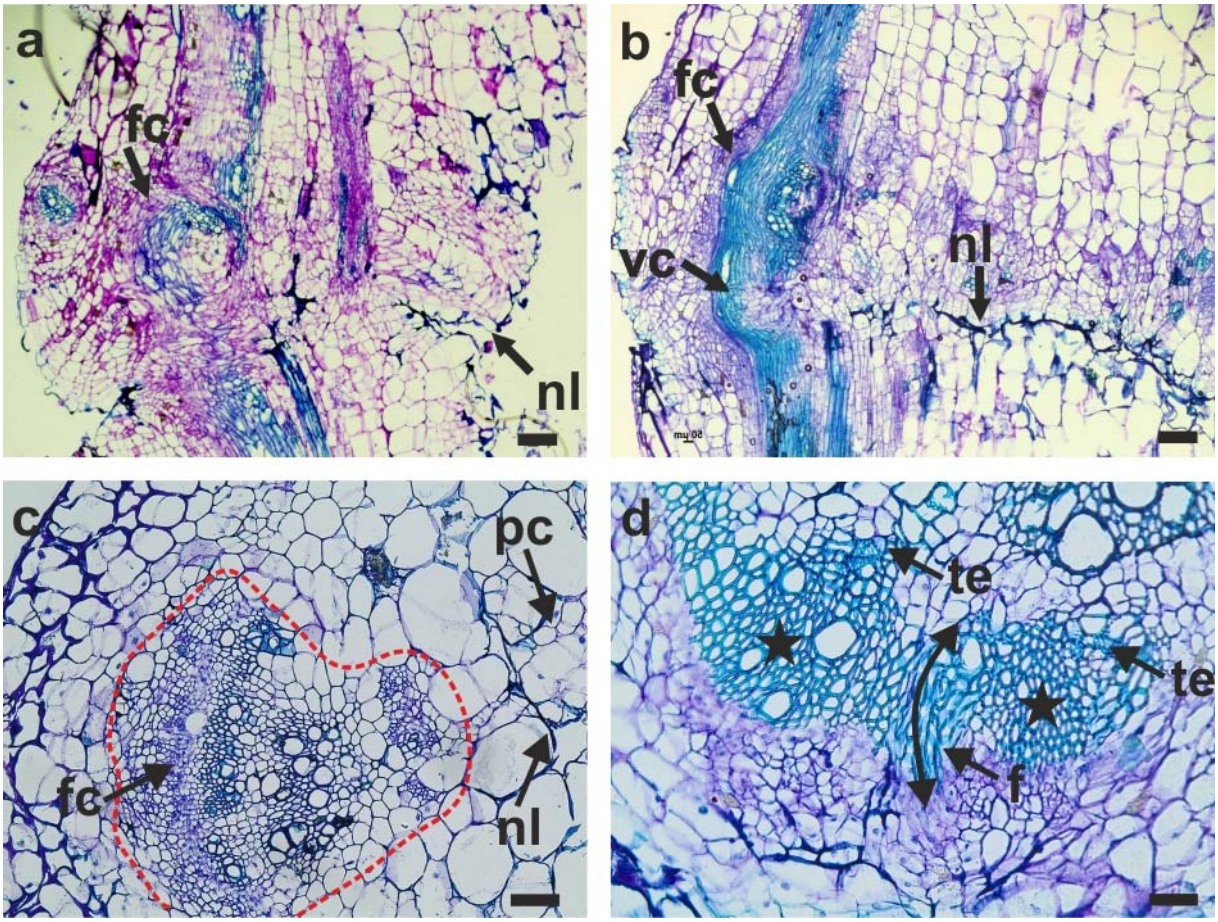

Fig. 5 Sections through the graft union area of a control plant (**a**) and juglone treated plants (**b–d**). Longitudinal (**a,b**) and cross (**c,d**) section. Note occurrence of callus in the pith area (**c**) and disturbed (**d**; double-headed arrow) and non-disturbed (**d**; asterisks) arrangement of xylem fibers and tracheary elements. fc – fascicular cambium; nl – necrotic layer; vc – vascular connection; dotted line – vascular bundle; pc – callus in the pith; te – tracheary elements. Scale bars: **a,b** 200 μm; **c** 100 μm; **d** 50 μm.

Discussion

The results presented here indicate that juglone at a 10^{-4} M concentration has positive effects on plant growth by increasing the interfascicular cambial cell divisions, the development of a continuous cambium layer along the stem circumference, and the development of fibers. Together, these phenomena resulted in an increase in the stem diameter. An increase in the shoot apical meristem activity, which was manifested by an increased number of developing leaves, was also detected. Moreover, the development of adventitious roots and visibly longer trichomes occurred in the juglone-treated plants. The obtained results indicate that juglone can have a positive effect on plant growth if the concentration is not lethal, and also, probably, if the time of its action is not too long.

In most of the cases reported in literature, the effects of juglone on plants have generally been toxic [16–18]. Juglone has been found to be an inhibitor of seed germination in cress, tomato, cucumber, alfalfa, radish, and watermelon [8,19–21]. A decrease in the growth of roots and seedlings that were under the influence of juglone was also described for many plants including soybean [22], corn [23], *Medicago* [24], cucumber [25], and also for some trees and shrubs [26].

In most earlier studies, the influence of juglone on seed germination, root growth, and sometimes also stem growth has been analyzed [22,25]. Analyses of the influence of juglone have also been the focus of studies on physiological parameters such as photosynthesis or mitochondria functioning and have indicated that juglone disturbs these physiological processes [3,4,22,27].

In the light of the above-mentioned studies, the results presented here, which show a positive influence of juglone on tomato plants, are rather unique. However, if we take into consideration the fact that allelochemicals can be beneficial in one species and harmful in another one and that this depends on the type and concentration of the allelochemicals and on the duration of the treatment [16–18], the growth stimulation that was observed in the presented studies is not improbable. At present, it appears that there are some plants that are tolerant to juglone [24]. It is possible that the concentration, duration of the treatment, and the way that juglone is applied may be important factors that change the reaction of plants to this allelochemical.

The juglone concentration seems to be very important in determining the harmful effect on plant growth. Studies on 16 plant species showed that this effect was different for seeds germination and plant growth depending on the juglone concentration [28]. It was shown that lower concentrations of juglone (within the range of 10^{-4} M to 10^{-6} M) enhanced germination in some species. In most experiments, a concentration of 10^{-3} M of juglone was very harmful for plant growth [28,29]. The same was observed in the studies presented here, which showed a positive influence of the lower juglone concentration on plant growth and a harmful influence of the higher concentration.

The duration of the juglone treatment also seems to be very important for determining its effect on plant growth. In growth analyses, the treatment usually lasted several weeks and the effect was harmful [19,21,28]. In experiments in which the physiological response or gene expression was investigated, the treatment with juglone lasted only hours [3,4,29,30]. Therefore, it is difficult to compare our results with most of the literature data. The only similar duration of juglone treatment was described in the case of two species of *Medicago* [24] and soybean [22]. The analysis of *Medicago* under the influence of juglone showed that at least one species, *M. lupulina*, was tolerant to this chemical after 7 days of treatment [24]. Thus, the results presented here are in accordance with at least these findings and suggest that juglone may have no harmful effect on plants [31].

Anatomical parameters such as vessel diameter, the number of stomata, or number of cell layers of the palisade parenchyma were analyzed in cucumber and it was shown that the number of palisade parenchyma layers increased under the influence of juglone. Moreover, it was determined that the cotyledons' mesophyll was much thicker in juglone-treated plants than in control plants [25]. In the results presented here, a stimulation of plant growth was also observed and it was caused by an increase in the cambial cell divisions, accelerated processes of cell differentiation and apical meristem activity, the latter manifested by an increase in the number of developing leaves and the presence of longer trichomes in comparison to control plants. Our results also showed the development of adventitious roots, which has not been mentioned in the literature as a result of the influence of juglone on plant growth (at least to the best of the knowledge of the authors). Such results may be explained as a plant's reaction to stressors. A plant's reaction to stress in the form of temporally increased cell division has been postulated in the literature [32–34]. Molecular analysis also showed that the response of a plant to stress conditions was correlated with an increase in the expression of stress response genes [24].

The reaction of a plant to different stressors is manifested by an increase in cell divisions. For example, in *Zea mays* cell divisions were stimulated in the distal elongation zone with lateral roots protruding after just 3 h [35]. In *Arabidopsis thaliana* under the influence of copper, the number of lateral roots increased [36]. Heavy metal stress can also induce a thickening of roots, including both increases in root density and root diameter [37,38]. As was postulated by Potters et al. [39], stress-induced morphogenic responses (SIMR) can be observed in plants that are exposed to a variety of distinct abiotic stressors and comprise three components: (***i***) inhibition of cell elongation, (***ii***) localized stimulation of cell divisions, and (***iii***) alterations in the cell differentiation status. The postulated hypothesis assumed that SIMRs are carefully coordinated stress acclimation responses rather than the unavoidable consequences of stress exposure and that plants use morphogenic responses to decrease their exposure to stress [39].

Conclusions

Although the morphology and histology of *Solanum lycopersicum* L. has been the subject of many studies, it has never been studied after treatment with juglone. The results obtained in the present study can be summarized as follows: (***i***) the control and juglone-treated plants differ in terms of histology; (***ii***) the effect of juglone depends on its concentration; and (***iii***) juglone at a concentration of 10^{-4} M stimulates plant growth by increasing the intensity of cambial cell divisions, the differentiation processes, and the acceleration of cell maturation.

Further experiments should be performed to test the effects of juglone for longer duration in order to determine whether the promotion of growth that is presented here is only temporary as a reaction of plants to stress conditions.

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Morfologiczno-histologiczna analiza łodyg pomidora (*Solanum lycopersicum* L.) traktowanego juglonem. Czy juglon wpływa pozytywnie na wzrost roślin?

Streszczenie

Badania prowadzono na kilkutygodniowych roślinach pomidora (*Solanum lycopersicum* L.) traktowanych juglonem w dwóch stężeniach (10^{-3} M lub 10^{-4} M) przez okres 7 dni. Juglon podawany był albo do systemu korzeniowego, albo w postaci pasty lanolinowej na dekapitowane pędy lub powierzchnię najmłodszego liścia. Sprawdzono również wpływ juglonu na tempo procesu zrastania się zrazu z podkładką po autoszczepieniu. Rośliny traktowane juglonem (niezależnie od miejsca aplikacji) w stężeniu 10^{-4} M, były większe w porównaniu do roślin kontrolnych, miały więcej rozwiniętych liści, charakteryzowały się wytwarzaniem korzeni przybyszowych, a proces zrastania się zrazu z podkładką przebiegał szybciej niż w roślinach kontrolnych. Juglon podany w wyższym stężeniu wpływał hamująco na wzrost i rozwój roślin w odniesieniu do analizowanych parametrów wzrostowych. Analiza histologiczna pokazała, że w roślinach traktowanych juglonem w stężeniu 10^{-4} M zwiększona była liczba komórek kambium w rzędach promieniowych, szybciej zakładał się ciągły na obwodzie łodygi pokład kambium i dochodziło do różnicowania się włókien, czego nie stwierdzono w roślinach kontrolnych, będących w tym samym wieku. Uzyskane wyniki zostały przedyskutowane w oparciu o literaturę opisującą wpływ juglonu na wzrost i rozwój roślin oraz w aspekcie odpowiedzi rośliny na działający czynnik stresowy.