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Originalarbeit

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Prevention of plant crown gall tumor development by the anti-malarial artesunate of *Artemisia annua*

Hemmung von Crown-Gall-Tumorwachstum durch anti-Malaria-aktives Artesunat aus *Artemisia annua*

Abstract

The antimalarial drug artesunate, a sesquiterpene trioxane lactone derivative of artemisinin from *Artemisia annua* L., is known for its extraordinary inhibitory effects on plasmodia and trematodes and also for suppressing the proliferation of human tumor cells. In the present study the effect of artesunate was investigated on rapidly dividing plant cells in *Agrobacterium tumefaciens*-induced crown gall tumor and wound callus cells at the model plant *Ricinus communis*. Low concentrations of artesunate (10 µM) were sufficient to completely suppress crown gall development upon permanent application. Within three weeks the shoots of artesunate-treated plants attained about double the size of the tumor-bearing plants and showed abundant, healthy and larger leaves. Moreover, artesunate retarded wound callus development and induced superficial necroses. However, artesunate did not prevent or inhibit infections of cucumber leaves by powdery (*Podosphaera xanthii*) or downy mildew (*Pseudoperonospora cubensis*). Young cucumber leaves showed symptoms of phytotoxicity upon treatment with very high artenusate concentrations of 100 µM and higher. Lower concentrations (50 µM or less) did not cause visible necrotic lesions. These novel findings suggest a general and conserved basic mode of action of artesunate in human, animal and plant cells, except of phytopathogenic fungi. A possible application of artesunate for biological control of crown gall development in grapevine and precious fruit trees is discussed.

Key words: *Agrobacterium tumefaciens*, crown gall, *Cucumis sativus*, downy mildew, growth promotion, powdery mildew, *Ricinus communis*, vascularization

Zusammenfassung

Die antimalaria-aktive Substanz Artesunat, ein Sesquiterpen-Trioxan-Lacton-Derivat von Artemisinin, ist für seine außergewöhnlichen Hemmeffekte auf Plasmodien und Trematoden sowie die Unterdrückung der Proliferation von Tumorzellen bekannt. In der vorliegenden Untersuchung wurde die Wirkung von Artesunat auf sich schnell teilende Pflanzenzellen von *Agrobacterium tumefaciens*-induzierten Crown-Gall-Tumoren und Wundkalluszellen bei der Modellpflanze *Ricinus communis* untersucht. Niedrige, aber ständig applizierte Artesunatkonzentrationen (10 µM) reichten aus, um die Crown-Gall-Entwicklung vollständig zu unterdrücken. Innerhalb von drei Wochen erreichten die infizierten, aber mit Artesunat behandelten Pflanzen etwa die doppelte Größe der tumorisierten Pflanzen und zeigten ein reichlicheres und gesundes Blattwerk mit größeren Blättern. Artesunat verlangsamte auch die Wundkallusentwicklung und verursachte oberflächliche Nekrosen. Es verhinderte jedoch nicht die Infektion von Gurkenblättern durch echten Mehltau, *Podosphaera xanthii,* oder durch falschen Mehltau, *Pseudoperonospora cubensis.* Junge Gurkenblätter reagierten auf sehr hohe Artesunatkonzentrationen, 100 µM und höher, mit Nekrosesymp-

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tomen, jedoch nicht auf geringere Konzentrationen von 50 µM und niedriger. Diese neuartigen Befunde lassen auf einen allgemeinen und grundlegenden Wirkungsmechanismus von Artesunat bei menschlichen, tierischen und pflanzlichen Zellen schließen, nicht aber bei phytopathogenen Pilzen. Eine mögliche Anwendung von Artesunat im Biologischen Pflanzenschutz zur Verhinderung von Crown-Gall-Tumoren bei Reben (Mauke) oder wertvollen Obstbäumen wird diskutiert.

Stichwörter: *Agrobacterium tumefaciens*, *Cucumis sativus*, Echter Mehltau, Falscher Mehltau, Mauke, *Ricinus communis*, Vaskularisierung, Wachstumsförderung

Introduction

Extracts of *Artemisia annua* L. have been used to cure chills and fever in China for more than 2000 years (Klayman, 1985). Searching for potent anti-malarial drugs, between 1972 to 1979 compounds of the glandular secretory trichomes of *A. annua*, such as the sesquiterpene trioxane lactone endoperoxide artemisinin and its derivatives including the most potent of them, artesunate, were identified as being active against malaria caused by *Plasmodium falciparum* and *P. vivax* infections (Klayman, 1985). It is active against chloroquine- and mefloquine-resistant strains with an excellent tolerance in patients and negligible side effects (Ribeiro and Olliaro, 1998; Ittarat et al., 2003). Since 2002, the World Health Organization (WHO) has recommended using these compounds as anti-malarial drugs, in particular against multidrug-resistant *Plasmodium* strains. In addition, in the mid 1990's the cytotoxicity of artemisinin against tumor cells was independently discovered by several research teams (Sun et al., 1992; Woerdenbag et al., 1993; Moore et al., 1995; Efferth et al., 1996). Evidence has been provided that artesunate is also active in cancer patients with uveal melanoma (Berger et al., 2005). Among other effects, artemisinins inhibit the angiogenesis of tumors (Chen et al., 2004; Dell'Eva et al., 2004; Huan-Huan et al., 2004).

Because artemisinin, a plant-derived compound, has amazing healing effects on taxonomically heterogeneous organisms such as protozoan plasmodia and animal/human cancer cells, it was an intriguing question, whether or not cells of higher plants and phytopathogenic fungi may also be affected. Until now only herbicidal effects of higher artesunate concentrations are described (Chen et al., 1991; Baghi et al., 1998; Bohren et al., 2004).

Agrobacterium tumefaciens or *A. vitis* induced plant tumors, namely crown galls, are feared world-wide in grapevines, pome fruit and cherry trees and sugar beet plants, and can cause up to 40% loss of the grape harvest in vineyards (Otten et al., 2008). Because the use of antibiotics is restricted, this disease has considerable economic impact and represents a challenge for plant protection research (Zeller and Ullrich, 2006). Strategies to control *A. vitis* crown gall development by application of non-pathogenic *A. vitis* antagonistic strains showed limited applicability in contrast to *A. radiobacter* strains K-84 and 1026 in the control of *A. tumefaciens* infections (Kerr and Htay, 1974; Staphorst et al., 1985; Burr and Reid, 1994; Bazzi et al., 1999; Creasap et al., 2005; Zäuner et al., 2006; Otten et al., 2008). By analogy to mammalian tumors, vascularization, the development of a dense net of vascular bundles, consisting of functional phloem and xylem strands, is a precondition for plant tumor development (Fig. 1a). Therefore, the aim of the present investigation was to test whether or not artesunate is also active against plant tumors. Disease control of crop plants by natural plant-derived agents is an increasing requirement for organic farming, such as the particularly efficient plant-derived extract of *Reynoutria sachalinensis* (Herger et al., 1988). Because of its efficacy in very divergent organismic kingdoms, the action of artesunate was studied also on the oomycete *Pseudoperonospora cubensis*, the downy mildew, and on the ascomycete *Podosphaera xanthii,* the powdery mildew, of cucumber.

Materials and Methods

Plant material

Castor bean (*Ricinus communis* L. var. gibsonii cv. Carmencita; Walz Samen, Stuttgart, Germany) was grown in standard potting soil LD 80 and kept in the greenhouse with 16 h light and 8 h dark cycles at 28°/20°C. 12d-old seedlings were wounded with razor blades 1 cm below the cotyledons by 2 longitudinal cuts of 5 mm length and inoculated with bacterial pellet.

Cucumber (*Cucumis sativus* L.) cv. Chinesische Schlange, was cultivated in LD 80 potting soil and kept in a growth chamber at 22°C and at 70% RH at 18 h light and 6 h dark. 3 weeks after sowing the 2 remaining first leaves, left over after removing side shoots, were pre-treated with artesunate (10 and 50 µM in 0.1% dimethylsulfoxide [DMSO]) or with 0.1% DMSO and 24 h later both leaves were inoculated with spores or sporangia.

Pathogens

The nopaline wild-type strain *Agrobacterium tumefaciens* (Smith and Townsend) C58, obtained from the Max-Planck-Institut Köln, Germany, was grown in yeast extract broth (YEB) as described earlier (Aloni et al., 1995).

For inoculation with powdery mildew, *Podosphaera xanthii* (Castagne), conidia from infected cucumber plants were collected and adjusted in 0.0125% Tween 20 in de-ionized water to a density of 1 x 10^4 spores ml^{-1}. Sporangia of downy mildew, *Pseudoperonospora cubensis* (Berk et Curt.), were adjusted to a density of 5 x 10^3 sporangia ml^{-1}.

Artesunate treatment

Ricinus: To investigate the effect of artesunate on tumor growth and wound callus development, artesunate was

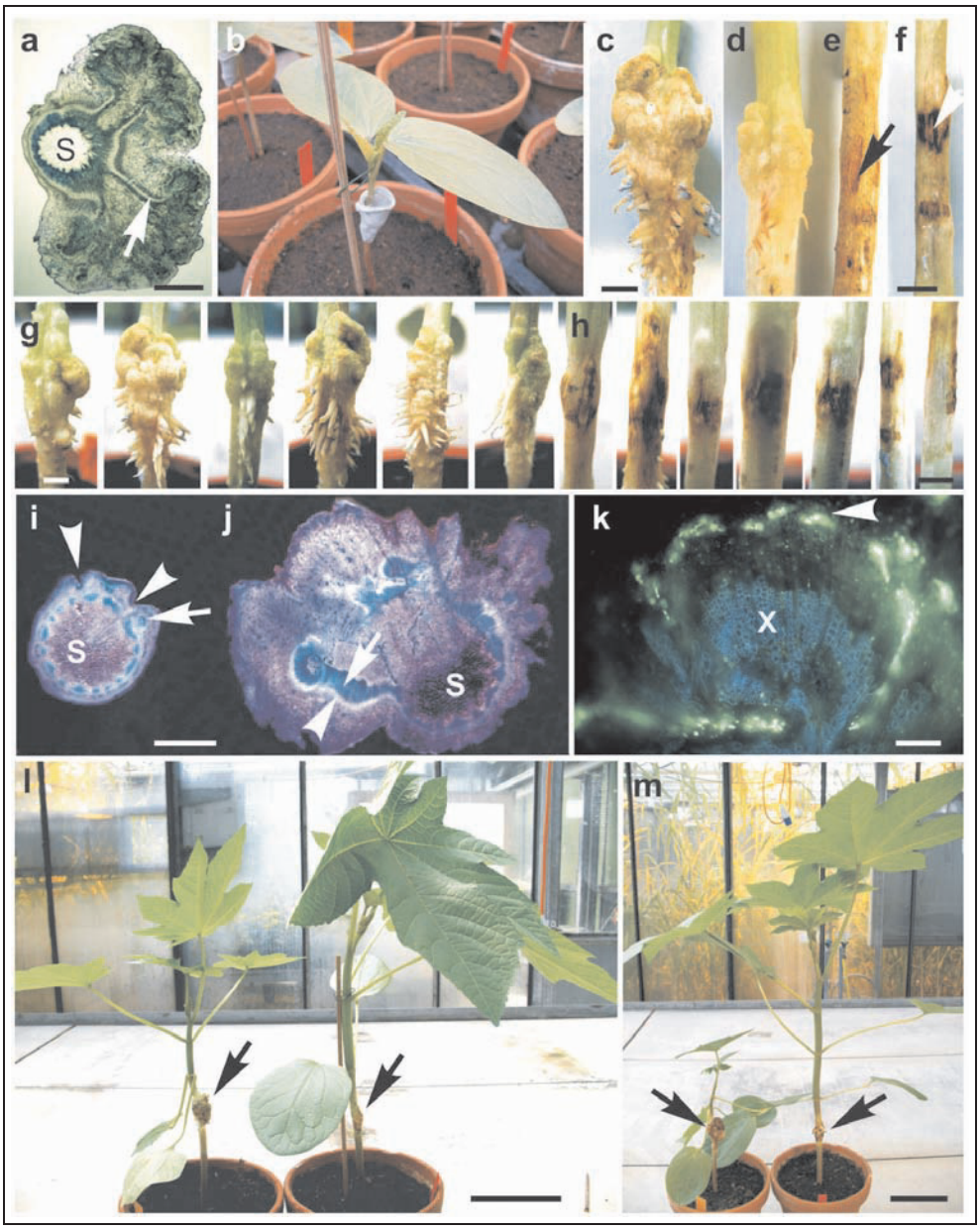

Fig. 1. Inhibition of plant tumor development by artesunate. **a** Vascularization (arrow) of a 4-week-old stem tumor of *R. communis*. **b** Application of artesunate (10 μM) with DMSO or only DMSO (0.1%) solution to *R. communis* seedlings in terostat funnels around the wound infected with *Agrobacterium tumefaciens*. **c, d, g** Tumor development in DMSO treated stems. **e, f, h** Absence of tumor proliferation upon artesunate treatment (arrows indicate the wound). **i** Stem cross-section of artesunate treated wound (arrowheads) with minute xylem differentiation (arrow, toluidine blue staining); **j** cross-section of tumor upon treatment with DMSO only, development of pathological cambium (arrowhead) and xylem (arrow). **k** Pathological vascular bundle differentiation with xylem (**X**) and phloem (arrowhead; aniline blue staining). **l** Plants 2 weeks and **m** 3 weeks post-inoculation, **left,** tumor development with only DMSO (arrow); **right**, upon artesunate treatment (arrow). **S** = host stem. Bars = 250 μm (**k**), 2.5 mm (**i, j**), 5 mm (**a, c – h**), 10 cm (**l, m**).

applied at the site of infection or wounding 1 d after inoculation or wounding. A Terostat funnel (Teroson, Heidelberg, Germany) was mounted around the wound and was kept filled up with 10 μM artesunate in 0.1% DMSO (Saokim Co. Ltd., Hanoi, Vietnam) or only 0.1% DMSO as control, following prior test series on the optimal effective concentration avoiding general phytotoxicity.

Cucumber: In preliminary experiments artesunate concentrations of 1 μM to 13 mM were tested on phytotoxicity in cucumber leaves. 100 μM and higher concentrations rapidly induced severe necroses in the younger leaves within 24 h (Fig. 2 j). Since leaves did not develop necrotic symptoms upon application of 50 μM artesunate and lower concentrations, in the further experiments 10 and 50 μM artesunate in 0.1% DMSO or 0.1% DMSO as control were protectively and thoroughly sprayed until run off on both the upper, adaxial, and the lower, abaxial, side of cucumber leaves using a glass atomizer (Desaga, Wiesloch, Germany). Treated plants were kept at 23°C for 24 h, and then spores of powdery mildew or sporangia of downy mildew were sprayed on the upper or the lower leaf sides. Plants inoculated with downy mildew sporangia were kept at 15°C in a humid and dark chamber for further 24 h and then at 20°C in a growth chamber for 14 d. Plants inoculated with spores of powdery mildew were transferred to the greenhouse and kept at 22°C for 12 d. All inoculated and control plants were daily thoroughly sprayed with artesunate or DMSO solution on both leaf sides.

Microscopy

After 2 to 3 weeks, 150 μm thick cross sections from tumor and wounded stem tissue were stained with 0.05% toluidine blue for 3 min or with 0.1% aniline blue (in 1 M glycine at pH 9.4) for 1 min and immediately viewed

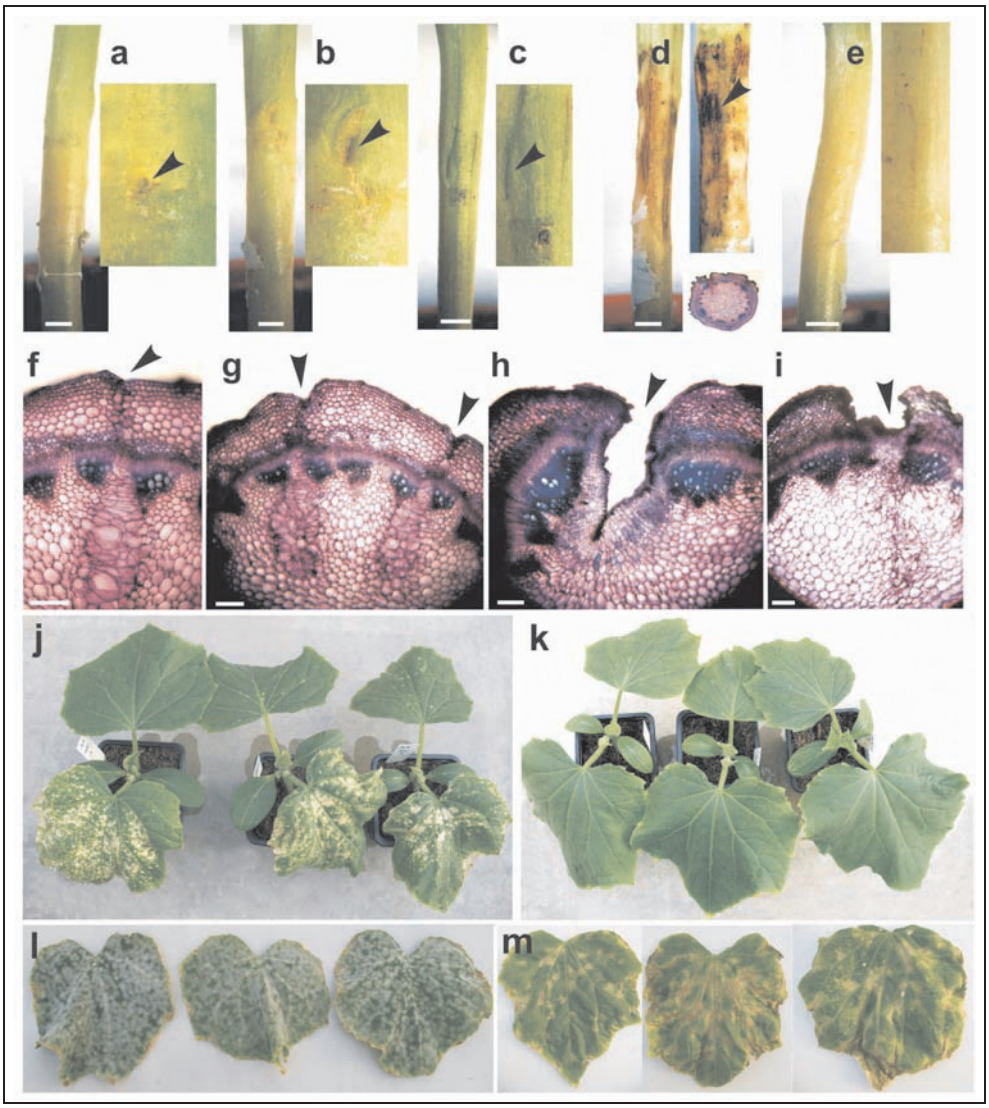

Fig. 2. Regeneration of wounded stems (arrowheads) in the presence or absence of artesunate without bacterial infection and effect of artesunate on powdery and downy mildew of cucumber. Treatment of *R. communis* stems with **a** DMSO, **b** dist. water, **c** wound without funnel, **d** artesunate, **e** artesunate without wounding. **f, g** Sections across the wound (arrows) two weeks after application of: **f** water, **g** DMSO, **h, i** artesunate, with necroses and restricted regeneration of wound callus. **(j)** Phytotoxicity of high artesunate concentrations (100 μM) on the younger front leaves of cucumber; slightly older leaves in the rear without necroses. **(k)** Lower artesunate concentrations (10 μM) induce no visible necroses on younger and older leaves. **(l)** Daily spraying of 0.1% DMSO only (left), 10 μM artesunate (middle) and 50 μM artesunate (right) do not prevent the severe development of powdery mildew infection (*Podosphaera xanthii*) and of **(m)** downy mildew infection (*Pseudoperonospora cubensis*). Bars = 500 μm **(f-i)**, 5 mm **(a-e)**.

with an Aristoplan epifluorescence microscope (Leica, Wetzlar, Germany) either under bright field or UV light (Leica, filter block A: excitation BP 340-380 nm, emission LP 430 nm). Micrographs were digitally reproduced from color slides taken with an Orthomat E camera system (Leica) on Kodak Ektachrome Elite 100 ASA daylight film.

Results

Stems of seedlings of *Ricinus communis* were used as an experimental model. They were wounded and infected with *Agrobacterium tumefaciens* strain C58. They rapidly developed crown galls upon the integration and expression of the T-DNA of the bacterial Ti plasmid (ALONI et al., 1995; ULLRICH and ALONI, 2000; ALONI and ULLRICH, 2008). To avoid washing out the bacteria and to ensure successful transformation of the plant tissue, artesunate (10 μM in 0.1% DMSO) was administered in terostat funnels mounted around the wound and infection site 24 h after inoculation of the stem tissue (Fig. 1b). Whereas the DMSO-treated control plants rapidly developed crown galls (Fig. 1c, d, g, j), artesunate caused only brown necrotic lesions around the wound (Fig. 1e, f, h). Microscopic analysis revealed strong vascularization within the DMSO-treated control tumors, consisting of considerable masses of xylem (Fig. 1j) and phloem (Fig. 1k, aniline blue staining). In artesunate-treated tissue, characteristic histological T-DNA-dependent phytohormone effects of auxin and cytokinin (VESELOV et al., 2003; ALONI and ULLRICH, 2008) became obvious in small vascular bundle development, proving successful transformation of the plant tissue (Fig. 1i). Hence the agrobacteria were not directly affected, which is in line with the fact that Gram-negative and Gram-positive bacteria are artemisinin-resistant (KLAYMAN et al., 1984). Importantly, further proliferation was inhibited when the wounds were carefully and permanently covered with artesunate solution. Already two weeks after inoculation the artesunate-treated plants were taller than the DMSO-treated tumor-bearing plants (Fig. 1l). Within three

weeks the shoots of artesunate-treated plants attained about double the size of the tumor-bearing plants and showed abundant, healthy and larger leaves (Fig. 1m).

Wounds of stems treated only with artesunate but not inoculated with *A. tumefaciens* developed characteristic brown necroses around the wound (Fig. 2d), in contrast to wounds treated in the same way with water or 0.1% DMSO or without any solution (Fig. 2a-c). However, unwounded tissue was not affected by artesunate (Fig. 2e). Microscopic analysis confirmed that in DMSO- or water-treated wounds, the wound callus regenerated the different tissues (Fig. 2f, g), while artesunate suppressed the complete regeneration of superficial wound callus cells and resulted in small necrotic cavities (Fig. 2h, i).

Artesunate induced necroses on younger cucumber leaves only at high concentrations (100 µM), but not on slightly older leaves. 50 µM and lower concentrations did not affect any healthy leaves (Fig. 2j, k). In spite of pre-treatment and continuous daily treatment with artesunate for 12 to 14 d growth of powdery and downy mildew was not inhibited, nor spore or sporangia germination, nor appressoria formation, nor the invasion of leaves by the fungal mycelium (Fig. 2l, m).

Discussion

The novel findings prove that artesunate, at a low concentration of 10 µM, exerts growth inhibitory effects on young proliferating plant tissues that either develop tumor or wound callus cells. In contrast, within three weeks the shoots of the artesunate-treated host plants, *R. communis*, attained about double the size of the tumor-bearing plants and showed abundant, healthy and larger leaves. The phenomenon that this sesquiterpene lactone endoperoxide severely affects organisms of completely different taxonomic groups such as viruses (HCMV, HBV, HCV, HSV), protozoa (*Plasmodium*), and trematodes (*Schistosoma*) (Klayman, 1985; Efferth et al., 2002; Utzinger et al., 2007), rapidly proliferating animal and human cancer cells (Efferth, 2007) and rapidly dividing higher plant cells, indicates a general mechanism of action. One of the mechanisms of action of artemisinin and its derivatives, known from plasmodia and cancer cells, is the induction of highly reactive oxygen species such as hydroxyl radicals, superoxide anions or carbon radicals, a reaction which is mediated by an excess of FeII ions (Moore et al., 1995; Efferth et al., 2004). Though artesunate affects the organisms of the aforementioned completely different taxonomic groups, spore or sporangia germination, appressoria formation and hyphal growth of the phytopathogenic fungi powdery and downy mildew remained unaffected. In this case an efficient detoxifying metabolic mechanism may be assumed, which raises problems also with many other known fungicides and biological agents.

Because of the similarities with so many other different organisms and the striking difference to phytopathogenic fungi, further detailed biochemical, molecular and physiological analyses, including the role of endogenous FeII ions in crown gall cells, are required to reveal the mode of action of artesunate in young differentiating plant cells, in particular in crown gall tissue. It is now important to examine the practical application of artesunate in grapevine crown gall prevention. Due to different anatomy and physiology of plants in contrast to animals and humans different application formulations have to be developed. Grapevine graftings or precious pome fruit trees may be protected or saved from crown gall disease by application of artesunate-containing wound dressing or pruning sealant formulations.

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