Phytotoxic Effects of Allelochemical Acacetin on Seed Germination and Seedling Growth of Selected Vegetables and Its Potential Physiological Mechanism

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Abstract: Acacetin is a naturally occurring flavonoid that displays multi-pharmacological activities, as well as phytotoxicity. In this study, seeds of four typical vegetables including lettuce (Lactuca sativa L.), radish (Raphanus sativus L.), onion (Allium cepa L.) and cucumber (Cucumis sativus L.) were selected to evaluate the phytotoxic effects of acacetin, and the model plant lettuce, which is also the most sensitive species to acacetin of the four vegetables, was used to research the phytotoxic mechanism of acacetin. Bioassays showed that the germination rate and germination potential of vegetable seeds were both decreased under a high concentration of acacetin. Acacetin displayed strong inhibitory effects on root growth, shoot growth and fresh weight of vegetable seedlings in a concentration dependent manner. After treatments with acacetin, the levels of O$_2^-$, H$_2$O$_2$, MDA, free proline and the number of dead cells in lettuce root tips were increased, while the mitosis index (MI) was decreased. These results indicated that acacetin could cause stress on lettuce seedlings and induce the accumulation of reactive oxygen species (ROS) in plant cells, leading to lipid peroxidation and then loss of cell viability and even cell death. Moreover, acacetin influenced the mitosis of the target plant, resulting in a decreased proportion of cells during the division phase. Together, acacetin showed strongly phytotoxic effects on vegetables, and the allelopathic activity mainly depended on the influence of ROS and mitosis of the receptor plant.

Keywords: acacetin; cell division; phytotoxicity; ROS; vegetable seeds

1. Introduction

Acacetin (5,7-dihydroxy-4′-methoxyflavone) is a naturally occurring flavonoid widely distributed in many higher plant species, such as Leptadenia reticulate, Ziziphora clinopodioides, Calea urticifolia, Chrysanthemum indicum, and Chromolaena odoratum [1–5]. Acacetin is the main active chemical in some traditional herb medicines. Research of modern medicines showed that acacetin displayed several biological activities including antimutagenic, antiperoxidant, anti-inflammatory and anticancer effects [6].

In addition to the pharmacological activities, acacetin also exhibited phytotoxic effects and acted as an allelochemical in several plant species. Acacetin was isolated from the aerial parts of wild oats (Avena fatua L.), and bioassays showed that the compound had significant allelopathic effects on the germination and seedling growth of wheat (Triticum aestivum L.). The inhibitory rates of acacetin on root length, shoot length and fresh weight were all more than 50% at 100 mg/kg. UHPLC-MS/MS analyses showed that acacetin could be released into the surrounding environment by leaching as a result of rain, dew or fog [7]. Acacetin was also isolated from Mexican propolis, and showed inhibitory activities on the root and shoot elongation of Lolium perenne and Echinochloa crusgalli seedlings, and
interfered with the photosynthetic electron flow [8]. The aqueous extracts of aerial and underground parts of *Fimbristylis miliacea* (L.) Vahl. inhibited the germination of *Emilia fosbergii* Nicolson seeds, and showed important chemical constituents including acacetin that can be directly related to the allelopathic effects observed in *E. fosbergii* seeds [9].

Acacetin was found in many plant species and showed strong phytotoxic activities [1–5, 7–9], indicating that acacetin had potential to be used as a herbicide in weed management. Moreover, the potential allelopathic effects of acacetin released by some invasive weeds such as *C. odoratum* could be a serious threat to crops [5]. Therefore, it is important to know the effects of acacetin on crops including vegetables. However, the detailed allelopathic effects and mechanism of acacetin on vegetables have not been studied.

In this study, four respective vegetable plants were used to evaluate the phytotoxic effects of acacetin, and the model plant lettuce was selected to study the mode of action of the phytotoxicity of acacetin.

2. Materials and Methods

2.1. Materials

The receptor vegetable plant species were selected according to previous studies [10, 11]. Seeds of lettuce (*Lactuca sativa* L.), radish (*Raphanus sativus* L.), onion (*Allium cepa* L.), and cucumber (*Cucumis sativus* L.) were kindly provided by Institute of Horticulture, Henan Academy of Agricultural Science. Acacetin (HPLC level) was purchased from Sigma-Aldrich Inc. Seeds of vegetables and acacetin powder were sealed and stored in darkness at 4 °C before use.

2.2. Bioassays

According to previous studies [12–17], the concentrations of acacetin used for bioassays ranged from 1 to 500 µM. Acacetin was dissolved in DMSO (Solarbio, Beijing, China) with concentrations of 500, 100, 50, 10 and 1 mM, respectively, as stock solutions. Vegetable seeds of similar sizes were surface sterilized with 70% ethanol for 1 min and washed with sterilized water 5 times. For seed germination, each petri dish containing a filter paper (Qualitative, Whatman-Xinhua, Hangzhou, China) had 3 µL stock solution and 2997 µL distilled H₂O added to make the final treated concentrations of 500, 100, 50, 10 and 1 µM, respectively. The same ratio (1‰, v/v) of DMSO in distilled H₂O was used as control. At least 50 seeds were transferred to the petri dish, and then incubated in a plant growth chamber (MGC-800HP-2, Yiheng, Shanghai, China) at 25 ± 1 °C in darkness. The numbers of the germinated seeds were recorded every day, and the germination rate and germination potential were calculated by the following formula:

Germination rate = (final number of germinated seeds/total number of the subjects’ seeds) × 100%

Germination potential = (number of germinated seeds on the germination peak period/total number of the subjects’ seeds) × 100%

For seedling growth, the sterilized seeds were transferred to a filter paper soaked with 3 mL distilled H₂O in a petri dish (Φ = 9 cm) and germinated at 25 ± 1 °C in a plant growth chamber in darkness. After germination, at least 6 seedlings of similar sizes were transferred to every well of a 6-well plate (NUNC, Shanghai, China), which contained various concentrations of acacetin and DMSO as control. After incubating at 25 ± 1 °C in darkness for 48 h, the root length, shoot length, and fresh weight of the seedlings were measured using a millimeter ruler. The relative growth of the seedlings was calculated as the ratio of treated groups to the control.

2.3. Cell Division

The chromosomes in cells of lettuce root tips were stained with Schiff’s reagent (Leagene, Beijing, China) according to Carvalho et al. [18]. Lettuce roots were fixed in Carnoy’s fluid (ethanol/acetic acid, 3/1, v/v) at 4 °C for 24 h, and then hydrolyzed using
5% HCl for 5 min. After staining with Schiff’s reagent for 30 min, root tips (about 2 mm) were excised and gently squashed, and then observed by a digital microscope (Yetech, Beijing, China). In each treatment, at least 1000 cells were observed and the numbers of cells in each division phase including prophase, metaphase, anaphase and telophase were counted. Mitosis index (MI) is the ratio between the number of cells during mitosis and the number of total observed cells.

2.4. Cell Viability

The cell viability in lettuce root tips were evaluated by double staining with FDA (12.5 µg/mL, MP Biomedicals, CA, USA) and PI (5.0 µg/mL, MP Biomedicals, CA, USA) according to Riaz et al. [19]. The stained roots were observed by a fluorescence microscope (Leica DMI4000B, Wetzlar, Germany) with excitation at 488 nm and emission >510 nm. At least 10 roots of each treatment were analyzed and the representative results are shown in the figures.

Root cell viability was also quantitatively evaluated by detecting the relative uptake of Evans blue (Solarbio, Beijing, China) according to Tamás et al. [20]. Root segments were stained with Evans blue and then soaked in N,N-dimethylformamide to extract the Evans blue that had been absorbed into the roots. Absorbance of the released Evans blue was measured at 600 nm by a spectrophotometer (DR6000, Hach, Loveland, CO, USA). The relative Evans blue uptake was calculated as the ratio between the OD values of the treated group and the control.

2.5. ROS Production

ROS production in root tips of the treated lettuce seedlings was estimated by staining with DHE (Solarbio, Beijing, China) according to Zhang et al. [21]. The fluorescence of the stained roots was analyzed by a fluorescence microscope (Leica DMI4000B, Wetzlar, Germany) with excitation at 488 nm and emission >510 nm. At least 10 roots per treatment were analyzed and the representative results are shown in the figures.

Endogenous H$_2$O$_2$ in roots of lettuce seedlings was quantitatively measured according to Velikova et al. [22]. After they were homogenized, centrifuged, and reacted with the reagent, the absorbance of lettuce roots was spectrophotometrically determined at 505 nm. The content of H$_2$O$_2$ in lettuce roots was compared with a standard graph and expressed in µM/g FW.

2.6. Lipid Peroxidation

The content of malondialdehyde (MDA) was measured to evaluate the lipid peroxidation. Lettuce roots (about 100 mg) were homogenized in 5 mL TCA (10%, w/v), and then centrifuged at 4000 × g for 10 min. The supernatant (1.0 mL) was added to 2.0 mL of TBA (0.6%, w/v) in 10% TCA. The tube containing the mixture was incubated in boiling water for 15 min, followed by an ice bath for 10 min. After centrifugation at 9000 × g for 5 min, the absorbance of the supernatant at 440, 532 and 600 nm was measured, and the levels of MDA were calculated according to Wu et al. [23].

2.7. Free Proline

Free proline was determined according to the method of Jabeen et al. [24]. Lettuce roots were homogenized in sulfosalicylic acid (3%, w/v) and then centrifuged. The supernatant was mixed with acid ninhydrin and glacial acetic acid at a ratio of 1:1:1, and incubated in boiling water for 1 h, and then in an ice bath for 10 min. Toluene (4.0 mL) was added to the mixture, and after the organic and inorganic phases were separated, the organic phase was monitored at 520 nm spectrophotometrically. Proline content was read from a calibration line constructed with pure proline (Alfa Aesar, Shanghai, China) standards.
2.8. Statistical Analyses

All experiments listed above were repeated at least three times and the results are represented as the means ± standard error (SE). SPSS 22.0 (SPSS Inc.; Chicago, IL, USA) was used for data statistical analysis. The differences between control and treated samples were determined using one-way ANOVA with an LSD test.

3. Results

3.1. The Influence of Acacetin on Seed Germination

Under the treatments of acacetin at 100 μM and less, the germination rates of the four vegetable seeds were similar with that of control. When the concentration of acacetin reached 500 μM, the final germination rate of lettuce and onion were significantly decreased. However, the germination rate of radish and cucumber were not affected by acacetin even at the highest concentration used (Figure 1A). At treatments of 1 and 10 μM acacetin, the germination potential of the four seeds were all similar to the control. The germination potential of lettuce and onion were strongly inhibited when the concentration of acacetin reached 50 and 100 μM, respectively. While, under 500 μM, the germination potential of all seeds was markedly decreased (Figure 1B).

![Germination rate (A) and germination potential (B) of four vegetable seeds under treatments with acacetin. The results presented are the mean of three replicates ± SE, different letters denote significant differences at p < 0.05 according to one-way ANOVA with an LSD test.](image)

3.2. The Influence of Acacetin on Seedling Growth of Four Vegetables

After treatment with 1 μM of acacetin, root growth of the vegetables did not show significant differences with control. When the concentration of acacetin reached 10 μM, the root growth of all seedlings was strongly inhibited except cucumber, whose root length significantly decreased only at the highest concentration used. The root length of seedlings showed a dose-effect response to acacetin concentrations, and the lettuce was most sensitive to acacetin with only 40% of the control at 500 μM (Figure 2A).

The stem length of all four seedlings remained the same as that of the control under low concentrations of acacetin at 1 and 10 μM. Acacetin significantly affected the stem length of onion and lettuce when the concentration reached 50 and 100 μM, respectively. The stem lengths of the four seedlings were largely reduced by acacetin at 500 μM (Figure 2B).

The fresh weight of the four vegetable seedlings gradually decreased with increased concentrations of acacetin. Lettuce and cucumber seedlings were more sensitive to acacetin with a significant reduction in biomass under 10 μM. Under the high concentrations of 100 and 500 μM, the fresh weight of all seedlings largely reduced (Figure 2C).
Figure 2. Seedling growth indices of root length (A), stem length (B) and fresh weight (C) of four vegetable seeds under treatments with acacetin. The results presented are mean of three replicates ± SE, different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.
3.3. Cell Division in Root Tips of Lettuce Seedlings after Acacetin Treatments

After treatments with acacetin, the mitosis processes of cells in lettuce root tips were disturbed. With the increasing acacetin concentrations, the mitotic index (MI) gradually decreased. At 50 μM and more, acacetin showed significant inhibitory effects on the MI of lettuce roots (Figure 3A). The ratio of cells during each phase were also influenced by acacetin, especially at relatively high concentrations of 50 μM and more (Figure 3B).

![Figure 3](image)

**Figure 3.** The mitotic index (A) and mitosis process (B) in root tip cells of lettuce seedlings after treatments with acacetin. The results presented are the mean of three replicates ± SE, different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

3.4. Cell Viability in Root Tips of Lettuce Seedlings after Acacetin Treatments

As shown in Figure 4, the red fluorescence of PI, which represents dead cells, was induced by acacetin in lettuce root tips under the concentration of 10 μM. Then, with the increasing acacetin concentration, the red fluorescence in the root tips was rapidly enhanced, indicating the number of dead cells was greatly increased.

![Figure 4](image)

**Figure 4.** Cell viability in lettuce root tips after treatments with acacetin. Lettuce seedlings were treated with acacetin at concentrations of (A) 0, (B) 1, (C) 10, (D) 50, (E) 100 and (F) 500 μM for 48 h. Roots were stained with FDA/PI. Green fluorescence and red fluorescence indicate viable and dead cells in root tips, respectively. Bar = 500 μm.

The ratios of dead cells in lettuce root were further quantified by determination of Evans blue uptake. Results showed that the relative uptake of Evans blue by lettuce roots was not notably influenced by acacetin at concentrations of 50 μM and less. However, when the concentration reached 100 and 500 μM, the relative uptake of Evans blue was significantly higher than the control (Figure 5).
Figure 4. Cell viability in lettuce root tips after treatments with acacetin. Lettuce seedlings were treated with acacetin at concentrations of (A) 0, (B) 1, (C) 10, (D) 50, (E) 100 and (F) 500 μM for 48 h. Roots were stained with FDA/PI. Green fluorescence and red fluorescence indicate viable and dead cells in root tips, respectively. Bar = 500 μm.

Figure 5. Relative Evans blue uptake of lettuce seedlings after treatments with acacetin. The results presented are mean of three replicates ± SE, different letters denote significant differences at \( p < 0.05 \) according to one-way ANOVA with an LSD test.

3.5. ROS Production and Lipid Peroxidation in Lettuce Seedlings after Treatments with Acacetin

After staining with DHE, the bright fluorescence, which mainly represents \( \text{O}_2^- \), was induced at 1 μM acacetin, while the fluorescence was enhanced at 10 μM acacetin. Then, with the increasing acacetin concentration, the fluorescence intensity in lettuce roots was rapidly enhanced. At high concentrations of 500 μM, there was very strong fluorescence in the treated roots (Figure 6).

Figure 6. ROS production in lettuce roots after treatments with acacetin. Lettuce seedlings were treated with acacetin at concentrations of (A) 0, (B) 1, (C) 10, (D) 50, (E) 100 and (F) 500 μM for 48 h. Roots were stained with DHE. Bright fluorescence shows ROS production (presumably \( \text{O}_2^- \)). Bar = 200 μm.

The level of \( \text{H}_2\text{O}_2 \) in treated roots was quantitated, and the results showed that acacetin has no significant effects on \( \text{H}_2\text{O}_2 \) content, at low concentrations of 1 and 10 μM. However, at concentrations of 50 μM or more, the content of \( \text{H}_2\text{O}_2 \) rapidly increased under acacetin stress (Figure 7).

Figure 7. \( \text{H}_2\text{O}_2 \) contents in roots of lettuce seedlings after treatments with acacetin. The results presented are mean of three replicates ± SE, different letters denote significant differences at \( p < 0.05 \) according to one-way ANOVA with an LSD test.
The level of H$_2$O$_2$ in treated roots was quantitated, and the results showed that acacetin has no significant effects on H$_2$O$_2$ content, at low concentrations of 1 and 10 $\mu$M. However, at concentrations of 50 $\mu$M or more, the content of H$_2$O$_2$ rapidly increased under acacetin stress (Figure 7).

Figure 7. H$_2$O$_2$ contents in roots of lettuce seedlings after treatments with acacetin. The results presented are mean of three replicates ± SE, different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

MDA, an end product of lipid peroxidation, was measured to evaluate the lipid peroxidation by excess ROS. As shown in Figure 8, the MDA level in lettuce roots was similar to that of the control under low concentrations of acacetin at 1 and 10 $\mu$M. Then, at 50 and 100 $\mu$M, the level of MDA was obviously higher than that of the control. At the highest concentration of 500 $\mu$M, the MDA content was significantly increased.

Figure 8. MDA content in roots of lettuce seedlings after treatments with acacetin. The results presented are mean of three replicates ± SE, different letters denote significant differences at $p < 0.05$ according to one-way ANOVA with an LSD test.

3.6. Effect of Acacetin on Proline Accumulation in Lettuce Seedling Roots

Free proline in lettuce seedlings was determined to evaluate the stress intensity by acacetin. After treatments with acacetin, free proline levels in lettuce roots were first slightly
decreased at 1 µM and then gradually increased at 10 µM and higher concentrations of acacetin. Under the highest concentration of 500 µM, free proline content was about 1.5 times that of the control (Figure 9).

Figure 9. Proline content in roots of lettuce seedlings after treatments with acacetin. The results presented are mean of three replicates ± SE, different letters denote significant differences at p < 0.05 according to one-way ANOVA with an LSD test.

4. Discussion

As an allelochemical, acacetin displayed inhibitory effects on seedling growth of several plant species, including weeds, in previous studies [7–9], indicating that acacetin has the potential to be used as a natural product-based herbicide. In this study, our results showed that the seed germination and seedling growth of lettuce, onion, radish, and cucumber were all influenced by exogenous application of acacetin. Compared with the germination rate, the germination potential of vegetable seeds was more sensitive to acacetin. For different seeds, the germination of lettuce and onion were more sensitive to acacetin. These results indicated that acacetin reduced the germination speed and prolonged the germination process of vegetable seeds. The root growth, shoot growth and fresh weight of vegetables seedlings were inhibited by acacetin in a concentration dependent manner. Lettuce was the most sensitive species to acacetin of the four vegetables. Acacetin displayed the typical action of an allelochemical [25], and inhibited the growth of both weeds and crops. Therefore, more work, such as chemical structure modification of acacetin, is needed to research the herbicidal application of acacetin. Moreover, acacetin is wildly distributed in many plant species, including weeds such as C. odoratum [5]. Therefore, acacetin may be released by these weeds to farmland and influence the growth of vegetables and other crops [26].

The phytotoxic effects of acacetin were evaluated in previous studies and this study. However, the mode of action of the phytotoxicity of acacetin has never been investigated. In plant cells, oxygen-containing groups with high biological activity named ROS, such as H$_2$O$_2$ and O$_2$−, are produced as byproducts of the normal metabolism of oxygen [27,28]. ROS act as molecular signals with important roles in plant growth and development, and the responses to biological and abiotic stresses [29]. However, overproduction of ROS in plant cells will cause oxidative damage to proteins, DNA and lipids, and induce programmed cell death (PCD) [30].

The influence of ROS in receptor plants is found to be involved in the phytotoxic effects of allelochemicals in previous studies [15–17,31–34], indicating that ROS play essential roles in allelochemical-induced stress in plants. In this study, we found that the
levels of $O_2^-$ and $H_2O_2$ in lettuce roots were gradually increased after treatments with acacetin with increasing concentrations, indicating that acacetin could efficiently induce the overproduction and accumulation of ROS in the target plant.

As a result of overproduction, high levels of ROS accumulated in plant cells and caused oxidative stress, which lead to lipid peroxidation in the cell membranes [30]. MDA, a marker for oxidative stress, is a highly reactive compound from lipid peroxidation of polyunsaturated fatty acids [35]. Our results showed that the contents of MDA in lettuce roots increased after treatments with acacetin, indicating that the phytotoxicity of acacetin involved lipid peroxidation and damage of membranes.

The permeability and fluidity of the lipid bilayer of membranes will be changed by peroxidation, leading to dramatic alternation of cell integrity and even cell death [36]. The results in this study showed that the cell viability in root tips of lettuce was decreased and the number of dead cells increased under the stress of acacetin. Together with the results of ROS and MDA, we propose that acacetin can induce ROS overproduction in lettuce roots, and then the excessive ROS can cause membrane disruption and loss of cell viability, as well as cell death. Therefore, ROS might play a central role in the phytotoxic effect of acacetin.

Root growth is largely dependent on the cell division of the root meristem. In this study, we found that the MI and the numbers of cells in all mitotic phases were decreased with acacetin treatments, indicating that acacetin prevent cells from interphase entering into mitosis. As an anti-cancer agent, acacetin exerts an antiproliferative effect by blocking cell cycle progression in cancer cells [37]. Thus, acacetin could disturb cell division in both plant and cancer cells, and the arrest of mitosis is one of phytotoxic mechanisms of acacetin on lettuce root growth.

Under environmental stresses, free proline will rapidly accumulate in plants. As a signaling molecule, proline has many functions, including osmotic adjustment, detoxification of ROS and protection of membrane integrity [38]. Therefore, proline is essential for plants’ recovery from stress. Previous studies showed that proline accumulation was associated with ROS production and oxidative stress under environmental stresses in plants [39,40]. In this study, a rise in proline level and accumulation of ROS appeared in lettuce roots under acacetin treatments, suggesting that acacetin induced a strong anti-stress response in lettuce, and ROS might be involved in the accumulation of proline to regulate acacetin-induced stress.

5. Conclusions

Acacetin showed significant inhibitory effects on seed germination and seedling growth in four vegetables, displaying strong phytotoxic activity. Acacetin induced ROS overproduction and accumulation in lettuce cells, and excessive ROS caused lipid peroxidation followed by reduction of root vitality and cell death. Moreover, the mitotic processes were affected and the proline level was increased by the acacetin application. Therefore, allelochemical acacetin exerted strong phytotoxicity on vegetables and the effects were related to the influence of ROS and cell division.

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