Toxicity and Tissue Accumulation Characteristics of the Herbicide Pendimethalin in Ginger (Zingiber Officinale Roscoe)

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Research

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Abstract

Background

Environmental health and food safety issues potentially caused by the dinitroaniline herbicide pendimethalin are a worldwide concern. Due to its importance for crop plants, the determination of possible toxicity and accumulation characteristics of pendimethalin in ginger should be determined.

Results

The toxicity response of ginger and tissue accumulation effects of pendimethalin on ginger biomass were studied by utilizing pendimethalin in a dose-response study. No significant effect on ginger biomass is observed when the concentration of pendimethalin used is less than 6.7 ppm, while > 10 ppm pendimethalin significantly reduces the biomass of ginger. This is attributed to root damage. The net photosynthetic rate of ginger when treated with 16.7 ppm pendimethalin is 11.37% lower than that of the control organisms, which is mainly caused by stomatal limitation. In addition, high-dose pendimethalin (16.7 ppm) causes the accumulation of reactive oxygen species (ROS) in ginger. The activity of superoxide dismutase (SOD) and peroxidase increases accordingly, maintaining the dynamic balance of ROS content. There is no significant effect on malondialdehyde levels or on membrane permeability. Pendimethalin has no significant effect on the expression of ginger α-tubulin mRNA. The damage of high-dose (16.7 ppm) pendimethalin to ginger is mainly caused by oxidative stress. Pendimethalin is significantly accumulated in ginger roots, but not rhizomes.

Conclusions

Because of this, application of pendimethalin to treat weeds in ginger fields may not pose a threat to human health.

1. Introduction And Background

Pesticides are widely used to improve agricultural production efficiency [1]. However, the effects of the increasing use of herbicides [2] and the environmental risk assessment of pesticide regulation are unknown, as they relate to the needs of sustainable food production [3]. All of these factors may exacerbate environmental pressures such as climate change and habitat destruction. Only 10-30% of the herbicide is absorbed by weeds and fixed by soil particles. Most herbicides will enter the groundwater or surface runoff with rainfall or irrigation, causing environmental pollution [4, 5]. Most importantly, the potential link between pesticide exposure and human disease has been increasing in strength [6, 7].

As a dinitroaniline herbicide, pendimethalin is a weed pre-emergent soil treatment agent, which has the advantages of high efficiency, low toxicity, and long effectiveness. Pendimethalin has a wide spectrum of weed control; it can effectively control annual grass weeds and some annual broadleaf weeds. However, due to its stability, lipophilicity, and soil adsorption characteristics, it poses potential risks to the environment, affecting ecology and human health [8, 9]. Dinitroaniline herbicides are also highly toxic to aquatic organisms and invertebrates [10]. In addition, the structure containing dinitroaniline can form a carcinogen
(nitrosamine), which is also potentially harmful to human health. The U.S. Environmental Protection Agency (U.S. E.P.A.) has classified pendimethalin as a persistent bioaccumulating toxic substance. In 2014, the Priorities Advisory Group of the International Agency for Research on Cancer (IARC) named pendimethalin as a high priority by pointing out that pendimethalin would cause oxidative emergency and inhibit the antioxidant system of cells [11]. In addition, exposure to pendimethalin increases the risk of pancreatic cancer [12].

Microtubules are important cellular components vital to the division of eukaryotic cells, intracellular transport and morphogenesis. In plant cells, microtubules are also the determinant for the orientation of cell division planes [13]. Microtubules are usually made of α-tubulin and β-tubulin, and the polymerization state of microtubules is dynamic [14]. There are two loop structures (the M-loop and the N-loop) in α-tubulin, of which eight amino acids are inserted into β-tubulin to stabilize the M-loop of α-tubulin. Pendimethalin chemically interferes with the N-loop (H1-S2) structure of α-tubulin, hindering the polymerization of microtubules [15]. Pendimethalin can thus inhibit the mitosis of plants. Plant resistance to dinitroaniline herbicides is usually caused by differences in their specific target sites, for example, the α-tubulin mutations of Thr-239-Ile and Met-268-Thr in *E. indica* and the mutations of Leu-136-Phe and Thr-239-Ile in *S. viridis* lead to the resistance of dinitroaniline herbicides [16, 17]. In recent years, novel mutation sites of anti-dinitroaniline herbicides have been discovered. The mutations of Val-202-Phe and Leu-125-Met/Leu-136-Phe in *Alopecurus aequalis* [18], and the Arg-243-Met/Lys mutation in rice [19] have been identified. The difference in the N-loop target site usually changes the surface electrostatic potential distribution and cavity structure of the interaction between α-tubulin and dinitroaniline herbicides [20].

Ginger is a widely grown crop plant in China, with a very large dedicated annual planting area of about 230 thousand hectares. Weed control in ginger fields has always been an important factor affecting labor efficiency. Most herbicides have a strong effect on ginger. Since pendimethalin is a less harmful herbicide on ginger, it is commonly used in its production. However, Huang et al. [21] showed that an extended period of pendimethalin exposure of ginger significantly reduced plant height, leaf number, stem thickness, and chlorophyll content. The purposes of this study are to identify the mechanism of toxicity of pendimethalin in ginger and to determine an appropriate concentration for its agricultural use. In view of the health risks caused by pendimethalin, it is also important to ascertain its physical distribution in ginger plants.

2. Methods

2.1. Experimental approach

The experiment was performed in the horticulture experiment centre of Shandong Agricultural University. Field soil that had not been planted with ginger for three years was selected, and precisely 8 kg of soil was placed into each pot (diameter: 25 cm, height: 30 cm). The ginger cultivar “Shannong 1” was sown into pots 81 days later, and weeds were removed from the soil surface. Pendimethalin herbicide with the trade name Dongtai (33% Effective concentration) was then used. The treatments were designed according to the dosage guideline (10.0 ppm): Varying concentrations (CK (0 ppm), PM1 (4.0 ppm), PM2 (6.7 ppm), PM3 (10.0 ppm), and PM4 (16.7 ppm)) of pendimethalin were evenly sprayed on the appropriate soil surface.
using a sprayer. Each treatment was repeated three times. The roots, leaves, and rhizomes of ginger were collected 112 days after sowing, washed in water, and then packed individually for storage at −80°C prior to subsequent measurements.

2.2 Analytical methods

2.2.1 Determination of photosynthetic system

The photosynthetic Rate (Pn), stomatal conductance (Gs), intercellular CO2 concentration (Ci), and transpiration rate (E) were measured using a portable photosynthesis system (Ciras-3, PP SYSTEMS, USA). All measurements were taken between 10:50 and 11:10 a.m. on August 25.

The photochemical quenching coefficient (qP), non-photochemical quenching coeffien (NPQ), quantum efficiency of PSII (qPSII), and variable fluorescence/fluorescence maximum (Fv/Fm) were measured according to Imaging-PAM chlorophyll fluorometer (HeinzWalz Gmb H, Effeltrich, Germany).

2.2.2 Determination of physiological index

Root activity was determined by using the triphenyl tetrazolium chloride (TTC) method [22]. Relative conductivity were determined according to Li et al. (2015). The α-tubulin content was determined using an α-tubulin detection kit (Jianglai), the procedure was carried out in strict accordance with the manufacturer's instructions [23].

2.2.3 Determination of antioxidant system

Superoxide dismutase (SOD), Peroxidase (POD), and Catalase (CAT) activity was measured according to Liu et al. [24]. Malondialdehyde (MDA), H2O2, and superoxide (O2−) were measured as described by Tang et al. [25].

2.2.4 Determination of pendimethalin

The roots, leaves, and rhizomes of ginger were freeze dried, ground and used for extraction. A quantity of 2 g of samples were extracted with 20 mL acetonitrile containing 1% acetic acid, with vigorous shaking, and 1 g of NaCl and 4 g of MgSO4 were added. After centrifuging for 5 min at 4000×g and clean-up using 0.6 g of MgSO4, 0.2 g of PSA, and 0.2 g of C18, 2 mL of supernatant was evaporated to dryness and reconstituted in 2 mL of methanol for UPLC-MS/MS analysis. The calibration curves (0.1-10 mg L−1 concentrations) for pendimethalin detection presented good linearity (R2=0.9896). The recovery of pendimethalin was 89.33 ± 1.24%.

The concentrations of pendimethalin in the supernatants were quantified using an LC-MS/MS system (TSQ Quantum Access Max™, Thermo, USA) equipped with an Acquity UPLC BEH C18 column (1.7 μm, 2.1×100 mm). The column was maintained at 40°C during sample analysis. The mobile phases were as follows: A: 2% methanol in water containing 0.05% formic acid (v/v); B: methanol containing 0.05% formic acid in water (v/v); The gradient was as follows: 0 min 10% B, 0.25 min 10% B, 7.00 min 100% B, 8.50 min 100% B,
8.51 min 100% B, 10 min 10% B. The flow of the column was set at a rate of 0.4 mL min\(^{-1}\). A 5 µL aliquot of extract was injected into the column. Antibiotics were measured in positive mode. The spray voltage and vaporizer temperature were maintained at 3.0 kV and 200 °C, respectively. Nitrogen was used as the sheath (20 arbitrary units) and auxiliary (40 arbitrary units) gas. Collision-induced dissociation was achieved with argon at a pressure of 1.5 mTorr. The retention times and ion transitions for LC-MS/MS analyses are provided in Table S1.

2.2.5 qRT-PCR

Total RNA was extracted using a RNA Isolation Kit (TianGen). A total of 400 ng RNA was reverse transcribed to cDNA using a cDNA synthesis kit (TianGen). qPCR was performed using the ABI Q6 (Thermo Fisher Scientific, United States) Real-Time PCR system. The target gene primers were designed using Primer Express 5.0, and the 28s gene was selected as the reference gene. Every primer was used 3 times. The relative expression levels were calculated using \(2^{-\Delta\Delta Ct}\) method, and the primers are shown in Table S2.

2.2.6 Protein structure prediction and analysis

These α-tubulin proteins of *Arabidopsis thaliana* and *Musa acuminata* sequences were retrieved from the national centre for biotechnology information (NCBI) database in FASTA format for further computational simulated investigation. The selected protein sequences were aligned using multiple sequence alignment clustalX2 software. Phylogenetic tree of α-tubulin sequences was analyzed using MEGA software with neighbourjoining method.

The amino acid composition, physical, and chemical characteristics of α-tubulin were evaluated using ProtParam tool (http://web.expasy.org/protparam/). The conserved domains of the proteins were analyzed using the CD-search tool (https://www.ncbi.nlm.nih.gov/cdd/). The transmembrane region of the proteins were analyzed using the Tmpred tool (http://www.ch.embnet.org/software/TMPRED_form.html).

The secondary structures of α-tubulin were predicted using web based server. The folding of protein directly depends on the number of helix, sheet, and turn of amino acid sequences in the secondary structure. Therefore, the presence of helix, sheet, and turn were predicted using PSIblast based secondary structure prediction (PSIPRED) (http://bioinf.cs.ucl.ac.uk/psipred/).

2.2.7 Statistical analysis

The physiological indicators were compared between different treatments using ANOVA. Differences were considered significant at \(p<0.05\). All statistical analyses were performed using SPSS 20.0 software. RDA was used to analyse with CANOCO version 4.5.

3. Results And Discussion

3.1 The physiological mechanism of ginger in response to the toxicity of pendimethalin

3.1.1 The effect of pendimethalin on ginger biomass
Pendimethalin has a dose-dependent effect on the growth of ginger. Under PM1 and PM2, there is no significant difference in plant height, stem diameter or rhizome weight compared with CK (Figure 1, P>0.05). As the concentration of pendimethalin increases, the growth of ginger is inhibited. Under PM4, the plant height, stem diameter, and rhizome weight are reduced by 28.73%, 8.78%, and 15.89%, respectively, compared with CK (Figure 1, P<0.05). This indicates that with an increased concentration of pendimethalin, the development of ginger is inhibited and could ultimately affect agricultural yield. However, pendimethalin has no significant effect on the number of shoots. There are known dose-dependent effects of other herbicides on various crops. Stephenson et al. [26] found that S-metolachlor damaged cotton, while pendimethalin had no such effect. Smith [27] found that low-dose pendimethalin had no significant effect on the biomass of *Basella alba*.

Root length is routinely used as a marker for toxicity of substances [28]. The root length of ginger under PM1 and PM2 is not significantly different from the control (Table 1, P>0.05), similar to the development of the aboveground tissues. Under the two higher doses, toxicity of pendimethalin to ginger root is demonstrated, and the root length decreases under PM3 and PM4. Under PM4, the root length decreases by 17.97% compared with CK (Table 1, P<0.05). A similar study was reported by Smith [29], who found that the root lengths of *Corchorus olitorius* and *Abelmoschus esculentus* seedlings treated with pendimethalin were significantly reduced. Similarly, under PM4, the root surface area, diameter, tips, and root weight of treated ginger plants are reduced by 28.68%, 8.69%, 6.91% and 19.17% compared with CK (Table 1, P<0.05), indicating that high-dose pendimethalin causes significant toxicity to ginger roots.

### 3.1.2 The effect of pendimethalin on the photosynthetic efficiency of ginger

Pendimethalin has an influence on ginger photosynthesis. Figure 2 shows that, except for PM4, pendimethalin has no significant effect on the net photosynthetic rate (Pn) of ginger (P>0.05). The Pn of ginger under PM4 is significantly reduced by 11.37% compared with the control (Figure 2, P<0.05), indicating that the root of ginger is more sensitive to pendimethalin than the leaves. On the other hand, pendimethalin accumulates only in the root, so that the ginger root system is more susceptible to pendimethalin poison than the leaves. Farhoudi and Lee [30] showed that pendimethalin could significantly reduce the photosynthetic rate of sunflower, while Jursík et al. [31] established that pendimethalin had no significant effect on the photosynthetic rate of lettuce. It may be that different crops have different tolerant thresholds for the toxicity of pendimethalin, or the decrease in the photosynthetic rate of ginger caused by pendimethalin is related to the inhibition of root development. In addition, the effects of pendimethalin on ginger transpiration rate, stomatal conductance, and intercellular CO$_2$ concentration follow the same trend as Pn (Figure 2), indicating that pendimethalin causes a stress response. The closure of stomata results in decreased transpiration rate and assimilated substrate (CO$_2$). This presumably leads to a decrease in Pn, mainly due to stomatal limitation. Wang et al. [32] pointed out that under stress, the closure of rice stomata is the main reason for the reduced photosynthetic rate. In addition, Li et al. [33] found that ABA-induced H$_2$O$_2$ production is related to the closure of stomata, which is also related to that PM4 increases the content of H$_2$O$_2$ in ginger leaves (Figure 4).
Chlorophyll fluorescence reflects the photosynthetic efficiency of plants and is measured to determine the degree of damage to plants [34]. The maximum quantum yield Fv/Fm of photosystem II (PSII) is correlated to the degree of damage to plant leaves [35, 36]. As shown by chlorophyll fluorescence images (Figure 3A), pendimethalin causes no obvious damage to ginger leaves. Under PM4 only, the Fv/Fm is 3.32% lower than that of the control (P<0.05), indicating that the critical toxicity concentration value for ginger to pendimethalin is between PM3 and PM4. Li et al. [37] found that pendimethalin did not affect the Fv/Fm of soybeans, but Shabana et al. [38] found that pendimethalin treatment significantly reduced the Fv/Fm of Protosiphon botryoides.

φPSII represents the non-cyclic electron transport efficiency of PSII, and qP reflects the reduction status of QA in the PSII reaction center. The changing trends of φPSII and qP in ginger leaves are similar to Fv/Fm, and under PM4, φPSII and qP decrease by 6.26% and 4.59%, respectively, compared with that of the control CK (Figure 3, P<0.05). The effect of pendimethalin on the PSII of soybeans is consistent with the results of this study [37]. Pendimethalin reduces the photosynthetic efficiency of ginger, and the reduction in linear electron transfer efficiency leads to the formation of ROS by processed light energy, which is related to the higher ROS content of ginger under PM4.

Non-photochemical quenching (NPQ) reflects the degree of heat dissipation of crops. This usually increases under abiotic stress [39]. Figure 3 shows that NPQ exhibits an opposite trend compared to Fv/Fm. Pendimethalin has no significant effect on NPQ of ginger leaves, except for PM4, which significantly increases NPQ by 6.99% compared with the control (P<0.05). Studies have found that herbicides could improve NPQ in plants [40, 41].

3.1.3 Effect of pendimethalin on the antioxidant system of ginger leaves

The formation and elimination of ROS in plants are usually in a balanced state, and the content of ROS increases when exposed to exogenous toxicity [42, 43]. The production of ROS can affect plants by damaging proteins and cells [44], causing membrane peroxidation [45], and affecting various metabolic pathways. Pendimethalin has no significant effect on the O₂⁻ content of ginger leaves (P>0.05), except that under PM4, H₂O₂ and O₂⁻ are significantly higher than the control by 27.89% and 19.58% (Figure 4, P<0.05). This indicates that low-dose pendimethalin does not reach the critical toxicity level for inducing ginger response, but under PM4, ginger begins to respond to the toxicity of pendimethalin, and the ROS produced is closely related to the closure of ginger stomata. Similar studies have shown that pendimethalin induced the production of ROS [46, 2].

Plants maintain a balance of ROS through generating antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) [47]. SOD, which catalyzes the conversion of free radicals to H₂O₂, is the first line of defense against ROS. Figure 4 shows that when the concentration of pendimethalin is increased, the SOD activity gradually increases. The accumulation of O₂⁻ in ginger is the main reason for increased SOD activity, and ginger can resist oxidative stress caused by antibiotics through increased SOD activity [48]. POD can catalyze the oxidation of substrates by H₂O₂ [49, 61], and it plays an important role in cell wall biosynthesis, lignification, and other cell functions [50, 62]. This study found that the change of
POD is similar to SOD, and it is significantly different from the control under PM2 (P<0.05). It shows that POD responds earlier than SOD in defending the toxicity of pendimethalin. In addition, during the early stages when pendimethalin affects ginger, the increased level of antioxidant enzyme activity could normalize the ROS content. Lehman et al. [59] used ROS as the indicator of cell toxicity. CAT can directly degrade H$_2$O$_2$ to H$_2$O, thereby reducing H$_2$O$_2$ damage [48]. This study found that pendimethalin has no significant effect on CAT activity (P>0.05), which may be related to the insensitivity of CAT to pendimethalin toxicity. It has been found that CAT behaved differently in different plants against external stress [51, 65, 66].

3.1.4 The effect of pendimethalin on root activity, MDA and relative conductivity

Root activity is the expression of plant absorption capacity. Under PM1 and PM2, no significant difference is found in root activity between the treatment and the control (P>0.05). Under PM3 and PM4, the root activity is significantly reduced, that of PM4 is 22.62 % lower than the control (Figure 5, P<0.05). A similar study was reported by Song et al. [52], who found that 742 g/hm$^2$ pendimethalin had no significant effect on the root activity of cotton, while 1113 g/hm$^2$ pendimethalin significantly inhibited its root activity, destroying the membrane structure.

Malondialdehyde (MDA) content reflects the degree of cell membrane peroxidation, and electrical conductivity reflects the degree of ion release after the cell membrane is damaged. Generally, when the plant is under external stress or toxicity, MDA content and conductivity will increase [53, 63]. In this study, pendimethalin has no significant effect on MDA and relative conductivity of ginger (P > 0.05). It has been demonstrated that herbicides can increase MDA and relative conductivity of plants [24, 54, 64], mainly because that herbicides damage plants. However, in this study, the critical value of pendimethalin toxicity to ginger is around PM3, and the effects of the toxicity have just begun to manifest. In order to resist the toxicity of pendimethalin, ginger reduces the excessive production of ROS by increasing the activity of antioxidant enzymes. The ROS content in this experiment is in a dynamic balance, the plasma membrane of ginger is not damaged, and the relative conductivity does not change significantly.

3.1.5 Correlation analysis shows that root development and the antioxidant system could be used as a sensitive indicator of the toxicity of pendimethalin in ginger.

First, the gradient length of each axis was estimated by detrended correspondence analysis (DCA), and then the canonical correspondence analysis (CCA) or redundancy analysis (RDA) was further selected. According to the principle of gradient length, >4.0 corresponded to CCA; 3.0 < gradient length < 4.0 corresponded to RDA or CCA; and gradient length < 3.0 corresponded to RDA. Based on the results of DCA analysis (maximum gradient length was 0.064), RDA was selected in this study. RDA analysis shows that the growth indexes (plant height, stem diameter and weight) of ginger are positively correlated with root structure (root length, root weight, surface area and tips) and photosynthetic indexes (Pn, Ci, E, Gs), negatively correlate with ROS, antioxidant enzyme (SOD, POD), MDA and relative conductivity, and had no significant correlation with CAT (Figure 6). It has been shown that root length can be used as a performance indicator of plant toxicity [28]. In this study, root length is also the most relevant indicator with among all ginger root growth
configurations. In addition, the SOD and POD of ginger are more sensitive to the toxicity of pendimethalin, which is the same as the results of this section. Therefore, the degree of ginger poisoning by pendimethalin can be assessed by the root development and the activities of SOD and POD of ginger.

3.2 Accumulation of pendimethalin in ginger and its optimal selection for weeding in ginger fields

Detection of pendimethalin residues in ginger roots, stems, leaves and rhizomes reveal that pendimethalin only accumulates in the roots. Jursik et al. [55] found that the residue of pendimethalin in lettuce increased with higher concentrations. This study has similar results. Pendimethalin is not detectable in the roots of PM1. When the concentration of pendimethalin is increased, the accumulation of pendimethalin in ginger roots gradually increases, and the highest residue identified is under PM4 (289.22 μg Kg⁻¹) (Figure 7). The octanol partition coefficient (LogKow = 5.18) of pendimethalin is higher, indicating that its water solubility is low, and it is not easily transferred from the root to the stem and leaves through passive transport. The accumulation of pendimethalin is mainly in the root. European Medicines Agency (EMA) defines the critical value of LogKow ≥ 4.5 for the phenomena of persistence, bioaccumulation, and the analysis of toxicity [56]. However, within a proper concentration range, there is no accumulation of pendimethalin in the edible organs (rhizome) of ginger. Therefore, the use of pendimethalin for weeding in ginger fields may have no health risk to humans.

There are significant differences in the weed removal effect of different concentrations of pendimethalin. Table 2 shows that there are no significant differences in herbicidal effects between different concentrations of pendimethalin at 5 days of treatment (P>0.05). After 10 days, there is no significant difference between PM2, PM3 and PM4 (P>0.05), but PM2 is significantly higher than PM1 (P<0.05). After 30 days, there is no significant difference in weed removal rate between PM2 and PM3 (P>0.05), but PM2 is significantly higher than PM1 and lower than PM4 (P<0.05). In view of the effect of pendimethalin on the growth of ginger, PM2 can be used as the optimal concentration of ginger for pre-emergent weed prevention, which has no significant effect on the development of ginger.

3.3 Bioinformatic analysis of ginger α-tubulin and its response to pendimethalin

*Musa acuminata* is a plant of Zingiberales and is the closest related species to ginger in the known genome database. We downloaded all α-tubulin gene sequences of *Musa acuminata* from NCBI, and then selected the sequences with P<10⁻⁵⁰ by local Blast from ginger transcriptome data (unpublished). Through conservative domain prediction, it is found that the selected genes have a predicted typical α-tubulin structure (Figure S1). Also, the α-tubulin gene of *Arabidopsis thaliana*, *Musa acuminata* and the selected ginger genes were examined by phylogenetic tree analysis. It was found that the genes were divided into 7 categories. CL17489.Contig1 was classified as Class I, with high homology to *AtTUA2*, Unigene28871 was classified as Class III; Unigene1894, Unigene39213, CL17215.Contig2 and Unigene33980 were classified as Class II, there was no predicted homologous α-tubulin gene of *Arabidopsis thaliana* and *Musa acuminata*; Unigene38694 and Unigene39214 are classified as Class VI, with higher homology to *AtTUAI*; CL7006.Contig1 is classified as Class VII with high homology to α-tubulin3 of *Musa Acuminata* (Figure S2).
Comparative analysis of nine selected ginger α-tubulin and \textit{AtTUA1} sequences (Table S3) found that \textit{AtTUA1} codes for a predicted 450 amino acid protein. From the nine predicted genes, α-tubulin amino acids in ginger are 188-448, the least is CL17215.Contig2, and the most is Unigene38694. The change in the trend of molecular weights is identical to the number of amino acids. The isoelectric point of \textit{AtTUA1} is 4.92, and the isoelectric points of the nine predicted ginger α-tubulin proteins are between 4.69 and 6.00.

Aliphatic index is often considered a measure of thermal stability of a protein [57], in this study, while the molecular weight of the protein increases, the aliphatic index tends to decrease. GRAVY indicates the hydrophilicity of a protein; a negative value indicates that the protein is a hydrophilic protein and a positive value indicates a hydrophobic protein. This study found that the predicted GRAVY of \textit{AtTUA1} is -0.194, and among the nine ginger α-tubulin sequences screened, eight had a negative predicted GRAVY, ranging from -0.204 to -0.028; only Unigene39213 has a predicted positive GRAVY (0.021).

Instability index indicates the stability of the protein; generally, the protein is predicted to be stable when the value is less than 40 and may be unstable when it is greater than 40 [58]. In this study, \textit{AtTUA1} has a predicted instability index of 40.92, which is an unstable protein. Unigene28871 (41.37), CL7006.Contig1 (42.43) and Unigene33980 (45.84) were all predicted to be unstable proteins; and Unigene1894 (34.40), Unigene39213 (33.24), CL17489.Contig1 (34.19), CL17215.Contig2 (23.14), Unigene38694 (38.26), and Unigene39214 (37.07) were all predicted as stable proteins.

Analysis of protein transmembrane structure (Figure S3) found that there is one predicted transmembrane structure in \textit{AtTUA1}, same as Unigene38694. Unigene28871 and Unigene33980 had no predicted transmembrane structure; the remaining ginger with predicted α-tubulin protein had one predicted transmembrane structure. The predicted secondary structure analysis (Table S4) of ginger α-tubulin and \textit{AtTUA1} protein found that only alpha helix, extended strand and random coil existed, and random coil accounts for the highest proportion, followed by alpha helix, with extended strand the least. Saboury et al. [60] found that alpha helix was positively correlated with protein stability. The alpha helix of the 9 predicted ginger α-tubulin proteins screened in this study are higher than those of \textit{Arabidopsis}, indicating that ginger α-tubulin has a higher stability, and at the same time providing evidence that ginger has a high resistance to pendimethalin.

According to the evolutionary relationship of ginger α-tubulin, one gene in each Class was selected for qRT-PCR analysis. Except for Unigene39213, pendimethalin has no significant effect on the transcription of α-tubulin mRNA in ginger (Figure 8, P>0.05), indicating that the effect of pendimethalin on ginger development is not caused by the different levels of α-tubulin. In addition, the determination of α-tubulin content in the root shows that pendimethalin has no significant effect (Figure 9, P>0.05), further indicating that the toxicity of pendimethalin to ginger is mainly due to oxidative stress, but not the effect of α-tubulin.

4. Conclusion

In this study, pendimethalin less than 6.7 ppm has no significant effect on the growth of ginger, while pendimethalin above 10 ppm significantly inhibits ginger growth. The toxicity of pendimethalin to ginger is not related to the expression and synthesis of α-tubulin. The damage to ginger induced by high
concentration of pendimethalin is mainly due to oxidative stress. This is correlated with the reduction of photosynthetic efficiency in ginger. The SOD and POD of ginger are more sensitive to the toxicity of pendimethalin, which can now be used as indicators for judging the toxicity of pendimethalin on ginger. Pendimethalin does not accumulate readily in the agriculturally important organs (rhizomes) of ginger, as it is only detected in the root, which may not pose a threat to human health.

**Declarations**

**Authors’ contributions**

Yao Lv: Data curation, Investigation, Writing - original draft. Yanyan Li: Validation, Software. Kun Xu: Resources, Writing - review & editing. Xiaohui Liu: Writing - review & editing.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

Materials and data are either described in the main manuscript or in the additional file. MATLAB codes are available on request.

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**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

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Tables

Table 1 The effect of pendimethalin on ginger root development.

| Treatment | Length (cm) | SurfArea (cm²) | AvgDiam (mm) | Tips | Weight (g)          |
|-----------|-------------|----------------|--------------|------|---------------------|
| CK        | 3760.20±28.19a | 1042.34±14.03a| 0.92±0.01a   | 5356.67±119.16ab | 18.47±1.31a         |
| PM1       | 3741.73±33.51a | 1040.91±32.87a| 0.92±0.01a   | 5417.67±67.80a    | 19.00±1.51a         |
| PM2       | 3652.72±62.93ab| 1013.11±22.18a| 0.86±0.01b   | 5362.67±115.74ab  | 17.90±0.51a         |
| PM3       | 3549.89±54.80b | 936.89±31.19b | 0.83±0.00c   | 5184.67±57.41bc   | 16.77±0.94ab        |
| PM4       | 3084.50±46.43c | 743.38±20.24c | 0.84±0.01bc  | 4986.33±42.32c    | 14.93±1.24b         |

Table 2 The weed removal effect of different concentrations of Pendimethalin.

|          | 5d     | 10d     | 20d     | 30d    |
|----------|--------|---------|---------|--------|
| PM1      | 0.83±0.24a | 0.64±0.10b | 0.50±0.08b | 0.56±0.08c |
| PM2      | 1.00±0.00a | 0.89±0.16a | 0.78±0.16a | 0.80±0.07b  |
| PM3      | 1.00±0.00a | 1.00±0.00a | 0.93±0.09a | 0.92±0.06ab |
| PM4      | 1.00±0.00a | 1.00±0.00a | 0.93±0.09a | 0.96±0.05a  |

Figures
Figure 1

The effect of pendimethalin on ginger biomass.
Figure 2

The response of ginger’s net photosynthetic rate (Pn), transpiration rate (E), stomatal conductance (Gs) and intercellular CO2 concentration (Ci) to pendimethalin.
Figure 3

Chlorophyll fluorescence response of ginger leaves to pendimethalin. (A) Chlorophyll fluorescence imaging of variable fluorescence/fluorescence maximum (Fv/Fm). (B) The acyclic electron transfer efficiency of PSII (φPSII). (C) The reflects the reduction degree of QA in the reaction center of PSII (qP). (D) The degree of heat dissipation (NPQ) under pendimethalin treatments.
Figure 4

ROS content and antioxidant enzyme activity in ginger leaves under pendimethalin treatments.
Figure 5

Root activity, MDA content and relative conductivity of leaves under pendimethalin treatments.
Figure 6

RDA-sort graph of root architecture, pendimethalin, photosynthesis and antioxidant system.
Figure 7
The content of pendimethalin in ginger root.
Figure 8

The transcript levels of α-tubulin of ginger.
Figure 9

The content of \( \alpha \)-tubulin in ginger root.

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