Allelopathic Stress Effects of Eupatorium adenophorum Extract on Three Weed Species (Postprint)

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Abstract

To investigate the effects of Eupatorium adenophorum extract on plant seed germination and early seedling growth and its underlying physiological mechanisms, three common field weeds—Echinochloa crusgalli, Chenopodium glaucum, and Amaranthus retroflexus—were used as experimental materials. Employing methods such as root aeroponic culture, we examined the influences of different concentrations of E. adenophorum extract on seed germination, seedling growth, root tip tissue structure, physiological characteristics of root border cells (RBCs), and root cap pectin methyl esterase (PME) activity in the three weed species. The results demonstrated that E. adenophorum extract exerted significant inhibitory effects on seed germination of all three plant species. Following treatment with 1000 mg/L E. adenophorum extract, the root tips of seedlings of the three weeds exhibited varying degrees of damage, including swelling, shrinkage, or deformation; the surface cells of the root tips detached, and the inner cells displayed disordered arrangements. Treatment with E. adenophorum extract significantly suppressed the number of RBCs at the root tips of seedlings of the three weeds (decreased by 44.5%, 48.3%, and 64.0% compared with the control, respectively); induced RBC apoptosis (with apoptosis rates reaching 81.7%, 91.3%, and 97.1%, respectively); and substantially increased the mucilage layer thickness of RBCs (increased by 99.0%, 65.5%, and 61.1% compared with the control, respectively) while inducing elevated PME activity. These findings indicate that E. adenophorum extract inhibited the production of root border cells in the three weeds and induced apoptosis of root tip border cells, thereby disrupting the protective system of root border cells for the root tip and ultimately inhibiting root growth and development. This study provides a theoretical basis for the development of E. adenophorum extract as a botanical herbicide.

Full Text

Physiological Mechanisms of Allelochemical Stress to Three Weed Species Caused by *Eupatorium adenophorum* Extracts

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Abstract

To investigate the effects of *Eupatorium adenophorum* extracts on seed germination and early seedling growth of plants and their underlying physiological mechanisms, three common field weeds—barnyard grass (*Echinochloa crusgalli*), oakleaf goosefoot (*Chenopodium glaucum*), and redroot pigweed (*Amaranthus retroflexus*)—were used as experimental materials. Using suspension culture and other methods, we examined the impacts of different concentrations of *E. adenophorum* extracts on seed germination, seedling growth, root tip tissue structure, root border cells (RBCs), physiological characteristics, and pectin methyl esterase (PME) activity in root caps of these three weed species. The results indicated that *E. adenophorum* extracts significantly inhibited seed germination and seedling growth of all three weeds. Following extract treatment, root tip surface cells detached, inner cells became disordered, and RBC numbers were significantly reduced. At 1000 mg/L, RBC numbers in *C. glaucum* and *A. retroflexus* decreased by 44.5%, 48.3%, and 64.0%, respectively, compared with controls. The extracts also induced RBC apoptosis, with apoptosis rates reaching 81.7%, 91.3%, and 97.1% for the three species, respectively. Additionally, *E. adenophorum* extracts significantly increased the thickness of RBC adhesive layers and enhanced PME activity. These findings demonstrate that *E. adenophorum* extracts inhibit RBC development, induce RBC apoptosis, and thereby disrupt the protective system of RBCs on root tips, ultimately inhibiting root growth and development. This study provides a theoretical basis for developing *E. adenophorum* extracts as botanical herbicides for green management of these three weed species.

Keywords: weed; *Eupatorium adenophorum* extracts; root border cells; allelochemical stress

Introduction

Eupatorium adenophorum is a perennial, shrubby invasive plant with strong allelopathic effects that has become a global malignant weed [1–3]. The plant is rich in natural bioactive compounds, and its extracts exhibit potent allelopathic activity against numerous plant species, inhibiting their growth and development, which suggests potential for development as botanical herbicides for green weed management. Previous studies have reported that *E. adenophorum* leaf aqueous extracts significantly inhibit seed germination and seedling growth of *Ixeris gracilis*, *Cyperus cyperoides*, *Chloris virgata*, white clover, and other species [4]. Wang et al. [5] found that *E. adenophorum* extracts at 100 mg/L significantly affected seed germination and seedling growth of white clover, *Macroptilium atropurpureum*, ryegrass, and alfalfa. Barnyard grass (*Echinochloa crusgalli*), oakleaf goosefoot (*Chenopodium glaucum*), and redroot pigweed (*Amaranthus retroflexus*) are among the most widely distributed and damaging weeds in the Poaceae, Chenopodiaceae, and Amaranthaceae families, respectively, causing serious problems in agricultural production across China. In recent years, barnyard grass has become the most pernicious weed in rice-producing regions, significantly affecting rice yield and quality. Current control methods primarily involve manual removal and chemical herbicides, which are labor-intensive and cause environmental damage [6–7]. Our previous research found that *E. adenophorum* extracts significantly inhibited seed germination and seedling growth of *A. retroflexus* and *C. glaucum*, and markedly affected reactive oxygen species metabolism in seedlings [8]. However, the cellular physiological mechanisms underlying these inhibitory effects remain unclear, and whether *E. adenophorum* extracts also exhibit allelopathic effects on barnyard grass has not been reported. Therefore, this study focused on these three major field weeds to investigate the physiological mechanisms of allelopathic stress caused by *E. adenophorum* extracts, particularly examining effects on root tips and root border cells at the cellular physiological level, to provide a theoretical basis for developing *E. adenophorum* extracts into botanical herbicides for green management of these weeds.

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1. Preparation of *Eupatorium adenophorum* Extracts

Mature leaves of *E. adenophorum* were collected, air-dried at room temperature, and ground into powder. The powder was extracted, and the extract was filtered using a sand core filter with a 0.45 m microporous membrane. The filtrate was combined and concentrated under reduced pressure using a rotary evaporator to obtain a thick extract. The extract was dissolved in an appropriate amount of water to create a suspension, which was then extracted with petroleum ether. The petroleum ether fraction was concentrated under reduced pressure and placed in a low-temperature vacuum drying oven to evaporate the petroleum ether, yielding the final *E. adenophorum* extract. An appropriate amount of extract was weighed and dissolved in a small volume of acetone (1

mL acetone per gram of extract), then diluted with water to prepare different concentrations of aqueous extract solutions for experimental use. Seeds of barnyard grass (*E. crusgalli*), oakleaf goosefoot (*C. glaucum*), and redroot pigweed (*A. retroflexus*) were collected from the Horticultural Station of Shanxi Agricultural University and stored in the dark at low temperature.

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2. Plant Cultivation and Extract Treatment

Uniform, plump seeds of the three weed species were selected and surface-sterilized with 0.1% HgCl for 5 minutes, then rinsed with distilled water to remove residual HgCl . For barnyard grass and oakleaf goosefoot, 50 seeds were used; for redroot pigweed, 30 seeds were used. The paper roll germination method was employed: seeds were evenly placed on 18 cm × 25 cm filter paper, covered with another filter paper of the same size, and carefully rolled into cylinders. Three paper rolls were bound together with rubber bands and placed in 300 mL beakers containing different concentrations of extract solutions, with distilled water treatment as control (Extracts Treatment, ET). Based on preliminary experiments showing differential sensitivity of the three weed species to extract stress, particularly redroot pigweed which showed seed rot at concentrations above 600 mg/L, the following concentrations were established: 150, 300, 450, and 600 mg/L for barnyard grass and oakleaf goosefoot; 250, 500, and 750 mg/L for redroot pigweed. Seed germination experiments were conducted in darkness at 25°C, with germination indices recorded daily.

For seed germination and seedling growth measurements, a higher concentration of 1000 mg/L ET was also included. For root tip observations, seedlings were grown until roots reached approximately 20 mm in length. For root border cell (RBC) measurements, uniform seedlings were selected and cultured using a hydroponic method: sterilized double-layer gauze was fixed on 500 mL beakers with rubber bands, 30 sterilized seeds were placed on the gauze, covered with filter paper and cotton, and 15 mL of extract solution at concentrations of 500, 750, or 1000 mg/L was evenly applied. The beakers were sealed with plastic wrap. After 5 days, when control seedling roots reached about 20 mm, root tips were excised for scanning electron microscopy sample preparation, RBC adhesive layer thickness measurement, and PME activity assays.

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3. Measurement Indicators and Methods

Seed Germination and Seedling Growth Indices: According to seed testing procedures [9], germination percentage (Gp = Ga/Gn × 100%, where Ga is the number of germinated seeds and Gn is the total number of tested seeds), germination potential, germination index, vigor index, shoot length inhibition rate, root length inhibition rate, and average fresh weight per seedling were measured. Ten seedlings were measured per treatment, with three replicates.

Root Tip Scanning Electron Microscopy: Referencing the method of Fan et al. [10], root tips (10 mm) from control and 1000 mg/L ET-treated seedlings were excised and fixed in 2.5% glutaraldehyde at 0–4°C for 4 hours. After washing with phosphate buffer (0.1 mol/L, pH 7.2), samples were dehydrated through an ethanol series (30%, 50%, 70%, 80%, 90%, 100%), 15 minutes per gradient, followed by two changes of 100% ethanol for 20 minutes each. Samples were then transferred to tert-butanol for 15 minutes, dried, mounted on specimen stubs with conductive tape, sputter-coated with platinum using a JEOL JFC-1600 ion sputter coater, and observed under a JEOL JEM-6490LV scanning electron microscope.

Root Border Cell Viability and In Situ Observation: Following the method of Hu et al. [11], five uniform root tips per treatment were soaked in 200 L distilled water in a centrifuge tube and shaken for 1 minute to separate border cells. The cell suspension was collected, transferred to a microsyringe, and centrifuged at 500 r/min for 3 minutes. After discarding the supernatant, 20 L of AO-EB stain (AO:EB = 1:1) was added, mixed, and 10 L was loaded onto a hemocytometer. Live cells (green fluorescence) and dead cells (orange-red fluorescence) were counted under a fluorescence microscope (Olympus BX53 with U-RFL-T, blue excitation). Viability rate was calculated as: (live cell count / total cell count) × 100%. For apoptosis detection, 75 L of cell suspension was stained with 50 L Hoechst-33258 for 5 minutes. Morphological characteristics of live and apoptotic cells were observed and counted under fluorescence microscopy [12].

RBC Adhesive Layer Thickness Measurement: Fifty microliters of cell suspension was mixed with 50 L India ink, dropped onto a slide, and observed under a fluorescence microscope. Thickness was measured at five different positions and averaged [13–14].

Root Cap PME Activity Assay: Following Hu et al. [11], 20 root tips (approximately 10 mm) were randomly excised from different treatments, ground in 400 L PME extraction buffer (0.2 mol/L Na HPO , 0.1 mol/L NaCl, pH 5.8) on ice, and transferred to a centrifuge tube. The mortar was rinsed with another 400 L extraction buffer and combined. After 20 minutes incubation on ice, samples were centrifuged at 4°C, 15,000 r/min for 10 minutes. The supernatant (300 L) was mixed with 0.5% (w/v) pectin solution containing 0.05% (w/v) bromothymol blue and 0.2 mol/L NaCl (pH 6.8), incubated at 37°C for 2 hours, and absorbance was measured at 525 nm. PME activity was calculated based on a standard curve and expressed as mol H /min.

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4. Data Analysis

All statistical analyses were performed using DPS 6.5 software. Significance differences among treatments were tested using Duncan' s new multiple range test at $P < 0.05$. Data were organized and plotted using Excel 2007, with Adobe

Photoshop CS5 used for image processing.

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1. Effects of Different Concentrations of *Eupatorium adenophorum* Extracts on Seed Germination and Seedling Growth of Three Weed Species

The results showed that *E. adenophorum* extracts at concentrations of 750 mg/L and above significantly inhibited seed germination and seedling growth of barnyard grass, with shoot length inhibition rate and root length inhibition rate reaching 24.7% and 6.8%, respectively. For oakleaf goosefoot, extracts at 250 mg/L and above significantly inhibited seed germination and seedling growth, reducing germination potential, vigor index, and seedling fresh weight by 29.4%, 31.2%, and 44.0%, respectively, with shoot and root length inhibition rates of 32.5% and 20.7%. For redroot pigweed, extracts at 150 mg/L and above significantly inhibited seed germination and growth, reducing germination potential and germination index by 23.3% and 30.2%, respectively, with shoot and root length inhibition rates of 5.1% and 12.7% at 150 mg/L, and 34.8% and 20.2% at higher concentrations. Comprehensive analysis revealed that the sensitivity of the three weeds to extract stress followed the order: redroot pigweed > oakleaf goosefoot > barnyard grass.

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2. Morphological Observation of Root Tip and Root Border Cell Damage Caused by *Eupatorium adenophorum* Extracts

Under scanning electron microscopy, root tip cells of control seedlings of all three weed species were tightly arranged with no damage. Extract treatment caused varying degrees of injury: root tip surface cells detached, inner cells became disordered and loose, and tissue structure was severely damaged. At 1000 mg/L, barnyard grass root tips showed swelling and deformation, oakleaf goosefoot root tips exhibited shrinkage, and redroot pigweed root tip structure was severely disrupted. Under light microscopy, control root tips of all three species showed numerous viable border cells attached to the root tip surface with intact structure. After 1000 mg/L extract treatment, border cell numbers decreased dramatically, and many root tip tissues and border cells were killed [FIGURE:2, FIGURE:3].

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3. Effects of Different Concentrations of *Eupatorium adenophorum* Extracts on RBC Number and Viability of Three Weed Species

Microscopic observation revealed that barnyard grass RBCs were mostly spindle-shaped, elliptical, or rod-shaped; oakleaf goosefoot RBCs were predominantly rod-shaped, crescent-shaped, or arc-shaped; and redroot pigweed RBCs were

generally larger. Live cells exhibited green fluorescence, while dead cells appeared red or orange-red. The results demonstrated that *E. adenophorum* extracts significantly inhibited RBC production, with stronger inhibition at higher concentrations. At 1000 mg/L, RBC numbers in barnyard grass, oakleaf goosefoot, and redroot pigweed decreased by 44.5%, 48.3%, and 64.0%, respectively [Figure 4A: see original paper]. The extracts also significantly reduced RBC viability, with viability rates decreasing by 92.2%, 62.4%, and 78.6% at 1000 mg/L [Figure 4B: see original paper].

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4. Effects of Different Concentrations of *Eupatorium adenophorum* Extracts on RBC Apoptosis Rates of Three Weed Species

In normal root tips of all three weeds, Hoechst-33258 staining revealed uniform blue nuclei with clear outlines and small blue bright spots. Apoptotic cells showed uneven coloration, patchy light blue fragments, or diffuse nuclear outlines with irregular large bright spots. Under extract stress, RBC apoptosis rates increased with concentration. At 500 mg/L, apoptosis rates in barnyard grass, oakleaf goosefoot, and redroot pigweed increased by 115.5%, 83.6%, and 26.7%, respectively, compared with controls. At 1000 mg/L, apoptosis rates reached 81.7%, 91.3%, and 97.1%, respectively [FIGURE:5, FIGURE:6]. Redroot pigweed showed relatively weaker tolerance to extract stress.

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5. Effects of Different Concentrations of *Eupatorium adenophorum* Extracts on RBC Adhesive Layer Thickness of Three Weed Species

The thickness of the RBC adhesive layer increased with extract concentration. At 500 mg/L, adhesive layer thickness in barnyard grass, oakleaf goosefoot, and redroot pigweed increased by 20.1%, 26.7%, and 17.4%, respectively. At 1000 mg/L, thickness increased by 99.0%, 65.5%, and 61.1%, respectively, all reaching significant differences [FIGURE:7, FIGURE:8]. The extract-induced thickening of the adhesive layer represents a protective response to allelochemical stress.

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6. Effects of Different Concentrations of *Eupatorium adenophorum* Extracts on Root Cap PME Activity of Three Weed Species

PME activity in barnyard grass showed no significant change with increasing extract concentration, while activities in oakleaf goosefoot and redroot pigweed increased with concentration. At 750 mg/L and 1000 mg/L, PME activity in redroot pigweed increased by 6.1% and 5.6%, respectively, reaching significant differences. At 1000 mg/L, PME activity in oakleaf goosefoot increased significantly by 5.6% compared with control. The relatively stable PME activity in

barnyard grass may be related to its lower sensitivity to extract stress [FIGURE:9, FIGURE:10].

Discussion

Cytotoxicity is a key characteristic of allelochemical action. Allelochemicals interact with receptor plant cell surfaces, disrupting internal cell structure, interfering with mitosis and gene expression, and ultimately inhibiting plant growth and development [15–17]. Our results demonstrate that *E. adenophorum* extracts exert cytotoxic effects on seed germination and seedling growth of the three weed species, with germination rate, vigor index, seedling fresh weight, and other parameters decreasing as extract concentration increased. Previous studies have shown that the main allelochemicals in *E. adenophorum*, such as eupatoriochromene and 2-deoxo-2-(acetyloxy)-9-oxo-10,11-dehydroageraphorone, cause rice root tip tissue disorganization [18]. In this study, high concentrations caused root tip swelling, surface cell detachment, and disordered inner cell arrangement in all three weeds, confirming the cytotoxic effects.

Root border cells are specialized cells that detach from the root cap and accumulate around the root tip. Once thought to be inactive [19], further research has shown that RBCs from many plants remain viable for extended periods after separation from the mother root [13]. We found significant interspecific variation in RBC number and viability among the three weeds, likely due to their different taxonomic families. The extracts significantly inhibited RBC development and induced apoptosis, as evidenced by AO-EB staining showing increasing numbers of red/orange cells and Hoechst-33258 staining revealing nuclear fragmentation and diffuse chromatin. These results confirm that allelochemical stress induces programmed cell death [12,21].

The RBC adhesive layer, composed of secreted chemical substances, protects root tips from toxic substances and microorganisms [22–23]. Under stress, plants increase adhesive layer thickness to adsorb or chelate toxins [24–25]. Our study showed that *E. adenophorum* extracts induced significant thickening of the adhesive layer in all three weeds, similar to responses observed in pea under aluminum stress [26] and maize under copper and allelochemical stress [11,27]. This thickening represents a protective physiological response.

PME activity regulates RBC release from the root cap by demethylating pectin and promoting pectin degradation [29]. While PME activity in barnyard grass remained relatively stable, activities in oakleaf goosefoot and redroot pigweed increased with extract concentration, possibly reflecting different stress tolerance mechanisms. The contrasting trends between PME activity and RBC number suggest that as root tip damage intensifies at higher concentrations, the capacity to produce RBCs declines. The more pronounced PME activity changes in the sensitive species (redroot pigweed and oakleaf goosefoot) compared to the tolerant species (barnyard grass) further support this relationship [30].

Conclusion

Eupatorium adenophorum extracts exert strong allelopathic stress on seed germination and seedling growth of barnyard grass, oakleaf goosefoot, and redroot pigweed. The allelochemicals inhibit RBC production, induce RBC apoptosis, damage root tip tissue and cellular structure, and disrupt the protective function of RBCs on root tips. This prevents normal transport of water and nutrients, ultimately inhibiting seed germination and early seedling growth. These results indicate that *E. adenophorum* extracts can be developed as botanical herbicides for green management of these three major weed species.

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Note: Figure translations are in progress. See original paper for figures.