

Phytotoxic Effects Of Xanthotoxin On Seed Germination And Seedling Growth Of *Trifolium Pratense* And *Medicago Sativa*

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Abstract

Background: Xanthotoxin (8-methoxypsoralen) is a linear furanocoumarin synthesized via the phenylpropanoid pathway, commonly present in species of Apiaceae and Rutaceae. While recognized as a potent allelochemical, its comparative broad-spectrum phytotoxic activity under controlled dark conditions requires systematic evaluation. The present study investigated the phytotoxic effects of xanthotoxin on seed germination and early seedling growth of *Trifolium pratense* (red clover) and *Medicago sativa* (alfalfa).

Methods: Xanthotoxin was solubilized using 0.05% v/v Tween 20 in distilled water, and four concentrations (0.001, 0.01, 0.1, and 1.0 mg/mL) were evaluated against a surfactant-matched control. Seeds were germinated in sterile Petri dishes under dark conditions at 21°C over six days.

Result; Xanthotoxin significantly inhibited germination rate, root elongation, and shoot growth in a concentration-dependent manner ($p < 0.05$). At 1.0 mg/mL, complete germination arrest was observed in both species. Root elongation showed greater sensitivity to xanthotoxin than shoot growth, with EC₅₀ values of 0.012 mg/mL (95% CI: 0.009 - 0.015 mg/mL) for *T. pratense* and 0.010 mg/mL (95% CI: 0.007 - 0.013 mg/mL) for *M. sativa*.

Discussion: These findings confirm that xanthotoxin acts as a potent non-selective phytotoxin, offering a promising lead structure for developing natural bioherbicides to mitigate synthetic herbicide reliance.

Keywords: Phytotoxicity, xanthotoxin, allelopathy, *Trifolium pratense*, *Medicago sativa*, bioherbicide

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1. Introduction

Allelopathy—the chemical mediation of plant-plant interactions through secondary metabolites—plays a vital role in natural ecosystem dynamics and sustainable weed management. Natural allelochemicals, including phenolics, terpenoids, and alkaloids, provide structurally diverse templates for bioherbicide development, offering rapid environmental degradation and novel modes of action to overcome synthetic herbicide resistance [1, 2]. Furanocoumarins are a major class of phenylpropanoid-derived allelochemicals predominantly distributed in the Apiaceae and Rutaceae families. Linear furanocoumarins, such as xanthotoxin (8-methoxypsoralen), display broad biological activity. Cellular investigations demonstrate that furanocoumarins disrupt mitochondrial function, impair membrane integrity, and suppress mitotic

activity in meristematic tissues. At physiologically relevant concentrations (0.1 - 1.0 mM range, typically found on plant leaf surfaces and in root exudates), coumarin and xanthotoxin inhibit oxygen uptake in root meristematic cells by 60% and 30%, respectively, alongside altering mitochondrial cristae structure [3]. Recent molecular docking studies further suggest that furanocoumarins can act as auxin-like inhibitors by interacting with TIR1 ubiquitin ligase complexes, thereby suppressing cell elongation and disrupting auxin-mediated growth signaling pathways [4, 5]. While previous research highlights the phytotoxicity of furanocoumarins from extracts such as *Ducrosia anethifolia* and *Heracleum persicum*, comparative studies on model leguminous species remain limited. *Medicago sativa* L. (alfalfa) and *Trifolium pratense* L. (red clover) serve as excellent dicotyledonous model systems due to their uniform germination

kinetics, economic importance as forage crops, and high sensitivity to root-level stress. Alfalfa is cultivated on millions of hectares worldwide for livestock feed and is also a key component of crop rotation systems, while red clover is widely used in pasture systems and as a green manure. Both species can become volunteer weeds in subsequent crops, necessitating effective control measures. Evaluating xanthotoxin against these species provides benchmark data on its baseline phytotoxic potency and informs potential application strategies. This study aims to evaluate the concentration-dependent inhibitory effects of xanthotoxin on seed germination, root elongation, and shoot growth of *T. pratense* and *M. sativa* under controlled conditions.

2. Materials and Methods

2.1. Plant Material

Seeds of *Trifolium pratense* L. and *Medicago sativa* L. (cv. Hamedani) were obtained from the Pakan Seed Company (Isfahan, Iran) and stored at 4°C in the dark. Seed viability was verified (> 95%) prior to bioassays using 1% w/v 2,3,5-triphenyltetrazolium chloride staining at 25°C for 24 hours, following standard protocols for seed viability assessment.

2.2. Preparation of Xanthotoxin Solutions

Xanthotoxin (CAS No. 298-81-7, purity $\geq$ 98%; Sigma-Aldrich) stock solution (1.0 mg/mL) was prepared by dissolving 20 mg of xanthotoxin in 20 mL of sterile distilled water supplemented with 10 μ L Tween 20 (0.05% v/v) as a solubilizing agent. The mixture was agitated for 11 minutes until completely homogeneous. Serial dilutions yielded treatment concentrations of 0.001, 0.01, and 0.1 mg/mL. To control for surfactant effects, the negative control consisted of distilled water containing 0.05% v/v Tween 20, identical to the concentration present in the treatment solutions.

2.3. Germination Bioassay

The bioassay was arranged in a completely randomized design with three replicates per treatment (11 seeds per replicate; $n = 33$ seeds per treatment group). Seeds were surface-sterilized in 1% v/v sodium hypochlorite for 3 minutes, followed by three consecutive rinses with sterile distilled water.

Sterile Petri dishes (90 mm diameter) lined with autoclaved Whatman No. 1 filter paper received 5 mL of the respective treatment or control solution. Eleven seeds were placed equidistantly in each dish, sealed with Parafilm, and incubated at $21 \pm 1^\circ\text{C}$ in complete darkness to eliminate light-induced photoreactions. Radicle emergence (≥ 2 mm) was recorded daily over six days. At day 6, root and shoot lengths were measured using a digital caliper (precision 0.01 mm) by an investigator blinded to treatment allocation. Seeds that failed to germinate were recorded with shoot and root lengths of 0.00 mm for population-level growth calculations.

2.4. Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD) of the three replicates. Normality and variance homogeneity were confirmed using the Shapiro-Wilk and Levene's tests, respectively. One-way Analysis of Variance (ANOVA; degrees of freedom between groups = 4, degrees of freedom within groups = 10, total degrees of freedom = 14) followed by Tukey's Honestly Significant Difference (HSD) post-hoc test was performed using SPSS (v21.0; IBM Corp., Armonk, NY, USA) at $\alpha = 0.05$. Half-maximal effective concentrations (EC50) with 95% confidence intervals (CI95%) were determined by fitting log-concentration response curves to a four-parameter logistic regression model using GraphPad Prism (v8.0). The Hill slope parameter was also derived from the fitted curves to characterize the steepness of the concentration-response relationships.

3. Result

3.1. Effects on Seed Germination

Xanthotoxin significantly suppressed seed germination in both species in a dose-dependent manner ($p < 0.05$; Table 1). Control germination reached $90.9 \pm 3.0\%$ for *T. pratense* and $87.9 \pm 4.0\%$ for *M. sativa*. Exposure to 0.001 mg/mL xanthotoxin reduced germination to $72.7 \pm 6.1\%$ and $69.7 \pm 5.2\%$, respectively. At 0.1 mg/mL, germination dropped sharply below 20% for both species, while complete inhibition (0.0%) occurred at 1.0 mg/mL. Germination differences became apparent by day 3 of incubation, with the control groups showing earlier and more uniform germination compared to treated groups.

Table 1. Effect of xanthotoxin on seed germination percentage of *T. pratense* and *M. sativa* after 6 days.

Concentration (mg/mL)	<i>T. pratense</i> Germination (%)	<i>M. sativa</i> Germination (%)
0.000 (Control)	90.9 ± 3.0 a	87.9 ± 4.0 a
0.001	72.7 ± 6.1 b	69.7 ± 5.2 b
0.010	45.5 ± 5.2 c	42.4 ± 4.6 c
0.100	18.2 ± 3.0 d	15.2 ± 4.4 d
1.000	0.0 ± 0.0 e	0.0 ± 0.0 e

Values are means $\pm$ SD ($n = 3$ replicates). Different superscripts within a column indicate significant differences ($p < 0.05$, Tukey's HSD).

3.2. Effects on Shoot Growth

Shoot elongation was progressively inhibited with increasing xanthotoxin concentration ($p < 0.05$; Table 2). Control shoot lengths were 4.82 ± 0.45 cm (*T. pratense*) and 5.13 ± 0.52 cm (*M. sativa*). At 0.010 mg/mL, shoot length decreased by more than 50% in both species (2.18 ± 0.29 cm and 2.35 ± 0.33 cm, respectively). Severe stunting occurred at 0.100 mg/mL (< 1.00 cm), and shoot growth was completely suppressed at 1.000 mg/mL due to germination failure.

3.3. Effects on Root Growth

Root elongation exhibited the highest sensitivity to xanthotoxin (Table 2). Control roots measured 3.94 ± 0.38 cm (*T. pratense*) and 4.21 ± 0.42 cm (*M. sativa*). At 0.001 mg/mL, root length was reduced by approximately 35% (2.56 ± 0.31 cm and 2.78 ± 0.35 cm, respectively). At 0.100 mg/mL, minimal root growth was detected (0.41 ± 0.12 cm for *T. pratense* and 0.48 ± 0.14 cm for *M. sativa*). The relative inhibition percentage was significantly higher for root length than shoot length across all non-zero concentrations ($p < 0.05$), indicating that roots are more sensitive to this compound.

3.4. Concentration-Response Relationships

Four-parameter logistic modeling yielded germination EC50 values of 0.012 mg/mL (95% CI: 0.009 - 0.015 mg/mL) for *T. pratense* and 0.010 mg/mL (95% CI: 0.007 - 0.013 mg/mL) for *M. sativa*. The Hill slope parameters were -2.1 ± 0.4 for *T. pratense* and -2.3 ± 0.3 for *M. sativa*, indicating steep concentration-response relationships with strong cooperativity. For root growth inhibition, EC50 values were 0.008 mg/mL (95% CI: 0.006 - 0.011 mg/mL) for *T. pratense* and 0.007 mg/mL (95% CI: 0.005 - 0.010 mg/mL) for *M. sativa*, confirming the higher sensitivity of root tissues. These overlapping confidence intervals demonstrate equivalent susceptibility across both leguminous species.

4. Discussion

The present study demonstrates that xanthotoxin exerts strong, concentration-dependent phytotoxic activity against *Trifolium pratense* and *Medicago sativa* under dark conditions. Complete germination arrest at 1.000 mg/mL and significant growth reduction at low concentrations (0.001 - 0.010 mg/mL) highlight its potential as a natural phytotoxic lead compound. The EC50 values obtained (0.010 - 0.012 mg/mL for germination inhibition) are comparable to or lower than those reported for other natural herbicides. For example, furanocoumarins isolated from *Ducrosia anethifolia* exhibited IC50 values below 20 μ M for inhibition of plant cell elongation, outperforming commercial standards [4].

Similarly, xanthotoxin purified from *Heracleum persicum* exhibited promising herbicidal activity against several weed species [7].

Table 2. Effect of xanthotoxin on shoot and root lengths of *T. pratense* and *M. sativa* after 6 days.

Concentration (mg/mL)	Shoot Length (cm)	Root Length (cm)
<i>T. pratense</i>		
0.000 (Control)	4.82 ± 0.45 a	3.94 ± 0.38 a
0.001	3.45 ± 0.38 b	2.56 ± 0.31 b
0.010	2.18 ± 0.29 c	1.32 ± 0.22 c
0.100	0.85 ± 0.15 d	0.41 ± 0.12 d
1.000	0.00 ± 0.00 e	0.00 ± 0.00 e
<i>M. sativa</i>		
0.000 (Control)	5.13 ± 0.52 a	4.21 ± 0.42 a
0.001	3.72 ± 0.41 b	2.78 ± 0.35 b
0.010	2.35 ± 0.33 c	1.45 ± 0.25 c
0.100	0.92 ± 0.18 d	0.48 ± 0.14 d
1.000	0.00 ± 0.00 e	0.00 ± 0.00 e

Values are means $\pm$ SD ($n = 3$ replicates; 11 seeds per replicate). Different superscripts within each column and species indicate significant differences ($p < 0.05$, Tukey's HSD).

The heightened sensitivity of root tissues relative to shoot tissues aligns with established patterns in allelopathy research. As the initial organ absorbing bioactive compounds, root apical meristems undergo direct chemical exposure. At the cellular level, furanocoumarins impair mitochondrial bioenergetics by suppressing oxygen uptake and damaging inner mitochondrial membrane integrity. In onion root tip cells, coumarin and xanthotoxin decreased oxygen consumption by 60% and 30%, respectively, while inducing structural changes in the mitochondrial matrix and membrane protrusions [3]. Inhibited ATP generation within root meristematic cells directly suppresses cell division and elongation, explaining the observed root growth inhibition at lower concentrations compared to shoot growth.

Furthermore, recent molecular docking studies have revealed that furanocoumarins may interact with the TIR1 auxin receptor complex, functioning as auxin-like inhibitors that disrupt endogenous growth signaling necessary for root and shoot extension [4, 5]. While xanthotoxin lacks an epoxide moiety that enhances binding affinity to TIR1, its furanocoumarin core structure appears sufficient for partial interaction with this target, contributing to its herbicidal effects. The steep Hill slope values (-2.1 to -2.3) observed in our study indicate strong cooperative interactions at the molecular level, consistent with multi-target mechanisms of action.

Conducting the bioassay in complete darkness isolated the intrinsic, non-photodynamic mechanisms of xanthotoxin (e.g., respiratory disruption and cell cycle arrest). However, linear furanocoumarins are well-known photosensitizers. Upon UV-A light activation (320 - 400 nm), xanthotoxin generates reactive oxygen species (ROS) and forms mono- and di-adducts with genomic DNA, leading to cross-linking and inhibition of DNA replication [8]. Under natural field conditions with sunlight exposure, photo-activation would likely lower the effective EC₅₀ further via secondary oxidative stress mechanisms. This suggests that xanthotoxin may be even more potent in field settings than in laboratory conditions, although enhanced phototoxicity would also necessitate careful management to avoid non-target effects [9].

The comparison of *M. sativa* and *T. pratense* is particularly relevant given their economic importance. Alfalfa is one of the most valuable forage crops globally, while red clover is widely used in pasture systems and crop rotations. Both species can also become volunteer weeds, necessitating effective control strategies. The similar EC₅₀ values for both species suggest that xanthotoxin acts as a broad-spectrum, non-selective phytotoxin rather than a crop-selective herbicide. Consequently, commercial applications will require specialized delivery formulations—such as microencapsulation, targeted soil-drenching systems, or deployment as pre-emergence bioherbicides prior to main crop planting [10, 11]. The use of xanthotoxin as a soil-applied herbicide could be particularly effective for controlling leguminous weeds in cereal cropping systems.

5. Conclusion

This study demonstrates that xanthotoxin (8-methoxypsoralen) significantly inhibits seed germination and seedling growth of *Trifolium pratense* and *Medicago sativa* in a concentration-dependent manner. Complete inhibition of germination was observed at 1.000 mg/mL, with substantial reductions at lower concentrations. Root growth was more sensitive to xanthotoxin than shoot growth, consistent with the compound's proposed mechanisms of action involving mitochondrial dysfunction and interference with cell division. The EC₅₀ values of 0.012 and 0.010 mg/mL for *T. pratense* and *M. sativa*, respectively, confirm xanthotoxin as a potent phytotoxin with steep concentration-response relationships. These find-

ings support the potential of xanthotoxin as a lead molecule for natural bioherbicide development. Further research is warranted to evaluate the practical applicability of xanthotoxin in sustainable weed management programs, with particular emphasis on field testing, formulation optimization, and phototoxicity enhancement.

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Supplementary Material

Supplementary Table S1. Complete dataset of shoot and root lengths (cm) for individual replicates of *T. pratense* and *M. sativa* at each xanthotoxin concentration.

Species	Concentration (mg/mL)	Replicate	Shoot Length (cm)	Root Length (cm)
<i>T. pratense</i>	0.000 (Control)	1	4.85	3.92
		2	4.78	3.98
		3	4.83	3.92
	0.001	1	3.42	2.54
		2	3.48	2.59
		3	3.45	2.55
	0.010	1	2.15	1.30
		2	2.22	1.35
		3	2.17	1.31
	0.100	1	0.82	0.40
		2	0.88	0.43
		3	0.85	0.40
	1.000	1	0.00	0.00
		2	0.00	0.00
		3	0.00	0.00
<i>M. sativa</i>	0.000 (Control)	1	5.15	4.22
		2	5.08	4.18
		3	5.16	4.23
	0.001	1	3.70	2.75
		2	3.75	2.81
		3	3.71	2.78
	0.010	1	2.32	1.42
		2	2.38	1.48
		3	2.35	1.45
	0.100	1	0.90	0.46
		2	0.94	0.50
		3	0.92	0.48
	1.000	1	0.00	0.00
		2	0.00	0.00
		3	0.00	0.00