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Thesis

INSIGHTS INTO PROFILING OF RESISTANCE MECHANISM OF ALFALFA TO ATRAZINE

Specialty 202 "Plant protection and quarantine".

20 Agricultural Science and Food production

for a Doctor Philosophy Degree (PhD)

The dissertation contains the results of own research. The use of ideas, results and texts of other authors are linked to the corresponding source.

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ANNOTATION

Zhu Yinghui. Insights into profiling of resistance mechanism of alfalfa to atrazine. - Manuscript Thesis for a Doctor Philosophy Degree (PhD): Specialty 202 “Plant protection and quarantine”. – Sumy National Agrarian University, Sumy, 2025

Medicago sativa L. is a type of forage grass belonging to the legume family, known as the "king of forage" due to its high nutritional value, excellent quality, and good palatability. Atrazine is a triazine herbicide and is the primary agent used to control corn fields in China. However, the long-term and frequent use of atrazine in the corn-to-alfalfa planting model will have apparent toxic effects on alfalfa in the soil residues, affecting the average growth and development. It needs further solutions in alfalfa production. This experiment aims to screen out alfalfa varieties with different resistances by identifying atrazine resistance in 60 varieties and taking resistant and sensitive varieties as research objects. Based on the perspective of non-target resistance mechanisms, alfalfa has different resistance to atrazine. The non-target resistance mechanism of atrazine was studied, and the differences in atrazine absorption and metabolism of alfalfa were further analyzed. A comparative analysis of the transcriptomes of resistant and sensitive alfalfa varieties exposed to atrazine was conducted to understand the mechanisms of resistance. The development of a novel and effective method for determining atrazine will allow for the rapid analysis of a significant number of samples for the determination of herbicide residues. Field experiments on the study of atrazine residues in soil and plants helped to establish the patterns of their accumulation. These studies provided a theoretical basis and practical solutions for breeding varieties in different regions agricultural production.

The resistance of 60 varieties of alfalfa to atrazine was analyzed with the detection of resistant (SF8001 (SF)) and sensitive (Juneng 2 (J2)) by the soil toxicity method. The experimental results showed that residual atrazine had a significant toxic effect on alfalfa, but different alfalfa varieties had different resistance to atrazine. The most resistant variety SF with an IC_{50} of 14.8750 mg/kg and the most sensitive J2 with

an IC_{50} of 0.2424 mg/kg were screened. The use of a hydroponic method to determine the inhibitory effect of atrazine on alfalfa varieties with different resistance showed that with increasing atrazine, the fresh weight inhibition rates of SF and J2 gradually increased, and the differences between the fresh weight inhibition rates were significant. The results of the herbicide toxicity study showed that the IC_{50} of the resistant variety (SF) was 8.28, and the IC_{50} of the sensitive variety (J2) was 0.29.

The effects of P450s inhibitor malathion and GSTs inhibitor NBD-Cl on the growth of SF and J2 were determined using hydroponic method. The results showed that the highest safe dose of malathion for SF and J2 was 25.00 mg/kg, and the highest safe dose of NBD-CI for SF and J2 - 0.25 mg/kg, with no significant difference compared to control.

The effects of malathion and NBD-Cl on the resistance levels of SF and J2 were determined using the hydroponic method. Compared with the use of atrazine alone, the IC_{50} values for a resistant and favorable variety were significantly reduced after combined treatment with malathion, with IC_{50} ratios of 3.02 and 2.21 for SF and J2 alfalfa, respectively. Therefore, it was speculated that the resistance of SF and J2 to atrazine may be related to P450s. Similarly, compared with the use of atrazine alone, the IC_{50} of SF and J2 decreased significantly after the combined treatment with NBD-Cl. The IC_{50} values of SF and J2 were 1.08 and 1.21, respectively. Therefore, it was speculated that the resistance of SF and J2 to atrazine may not be related to GSTs.

When studying the effect of atrazine stress on plant height (PH), root length (RL), shoot dry weight (SDW), and root dry weight (RDW) of varieties with different resistance, the following patterns were established: with increasing atrazine application concentration, these indicators decreased, the difference in the suppression of SF and J2 was significant, and the atrazine-sensitive variety was suppressed more strongly than the resistant one. When the concentration of atrazine was 2.0 mg/kg, SF decreased PH, RL, SDW, and RDW by 50.84%, 55.03%, 44.42%, and 54.13%, respectively, under atrazine stress. J2 reduced these indicators by 81.84%, 81.73%, 92.31%, and 85.00%, respectively. The results showed that the resistance of SF and J2 to atrazine was related

to photosynthesis.

The study of the effect of atrazine stress on the photosynthetic characteristics of SF and J2 showed that with increasing atrazine concentration in the soil, the net photosynthesis rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr) gradually decreased, while the intercellular carbon dioxide concentration (Ci) gradually increased. At a concentration of 2.00 mg/kg of atrazine, the Pn, Gs, Tr, and Ci of J2 decreased by 83.42%, 84.15%, 91.57%, and 46.69%, respectively. At 0.5 mg/kg, the Pn, Gs, and Tr of SF significantly decreased by 34.03%, 17.90%, and 27.59%, respectively, while Ci increased by 16.38% and showed significant differences compared to J2.

Determination of the effect of atrazine stress on the fluorescence parameters of chloroplasts of varieties with different resistance showed that with an increase in the residual amount of atrazine maximum quantum yield (Fv/Fm), maximum photochemical efficiency (Fv/Fo), actual photosynthetic efficiency (Y(II)), photosynthetic electron transport rate (ETR), and coefficient of photochemical fluorescence quenching (qP) all decreased, while non-photochemical quenching coefficient (NPQ) was the opposite. At a concentration of 2.00 mg/kg of atrazine, Fv/Fm, Fv/Fo, Y (II), ETR, and qP decreased by 11.76%, 76.90%, 80.00%, 63.12%, and 46.44%, respectively, while NPQ significantly increased by 37.64% compared to the control at this concentration. When the concentration of atrazine was 0.50 mg/kg, the Fv/Fm, Fv/Fo, Y (II), ETR, and qP of SF decreased by 10.11%, 8.00%, 24.56%, 26.62%, and 11.11%, respectively, while NPQ increased by 19.72%, which was significantly different from J2.

The chlorophyll and MDA (Malondialdehyde) contents of SF and J2 were both affected after atrazine stress. The results showed that with the increase of application days, the content chlorophyll *a* and *b* in SF remained higher than that in J2. Compared with the control, on the 10th day, the content of chlorophyll *a* in SF and J2 was 1.35 times and 4.53 times that of the control, respectively, and the content of chlorophyll *b* was 3.4 times and 1.06 times that of the control, with significant differences between

the two levels. The MDA content of SF remained lower than J2 with increasing application days. On the third day, the MDA content of J2 was 4.19 times that of the control and 1.91 times that of SF, with a slightly slower growth rate after 3 days. The resistance of SF and J2 to atrazine was related to the activity of defense enzyme systems.

The conditions of the UPLC-MS/MS method for the determination of atrazine in alfalfa plants were optimized. Differences in atrazine absorption and metabolism between SF and J2 were evaluated using this method. The results showed that the residual amount of atrazine in J2 was always higher than that in SF, and there was a significant difference. SF had a significantly higher metabolic rate of atrazine than J2. The emergence of resistance to herbicide in alfalfa may be related to the increased level of resistance absorption and metabolic capacity.

Comparative analysis of SF and J2 genes altered under atrazine stress using transcriptome sequencing technology showed that under the treatment of atrazine, there were 4032 differentially expressed genes in the aboveground part between J2-T and SF-T, of which 2233 genes were up-regulated and 1799 genes were down regulated. The GO and KEGG annotation analysis results indicated that differentially expressed genes were mainly involved in photosynthetic carbon utilization and metabolism, amino acid synthesis and UDP galactosyltransferase.

Determination of atrazine residues in soil and plants during two alfalfa growing seasons (2022-2024) showed a threat to plant growth. The amount of atrazine in the soil gradually decreased and then stabilized. The residual amount in SF and J2 varieties first increased and then decreased.

Keywords: *alfalfa, legumes, atrazine, toxicity, means of protection, protection measures, harmfulness, pesticide residuals, plant development, seedlings, transcriptome, breeding, variety, genotype prediction*

АНОТАЦІЯ

Чжу Іньхуей. Профілювання механізму стійкості люцерни до атразину. - Рукопис дисертації на здобуття наукового ступеня доктора філософії (PhD): спеціальність 202 «Захист і карантин рослин». – Сумський національний аграрний університет, Суми, 2025

Medicago sativa L. – вид кормової культури родини бобових, відомий як «король кормів» завдяки своїй високій харчовій цінності, відмінній якості та гарним смаковим якостям. Атразин відносять до групи триазинів і застосовують для контролю бур'янів у кукурудзяних полях Китаю. Однак тривале та часте використання цього гербіциду у сівозміні з люцерною має очевидний токсичний вплив на останню, впливаючи на середній ріст і розвиток рослин. Негативний вплив атразину вимагає подальших рішень у виробництві люцерни. Вивчення стійкості 60 сортів люцерни до атразину дозволило виявити стійкий та чутливий сорти як подальші об'єкти для дослідження. Виходячи з точки зору механізмів нецільової стійкості, люцерна має різну стійкість до атразину. Було вивчено нецільовий механізм резистентності до гербіциду та додатково проаналізовано відмінності в абсорбції атразину та метаболізмі люцерни. Провели порівняльний аналіз транскриптомів стійкого і чутливого сортів люцерни під впливом атразину для розуміння механізмів резистентності. Розробка сучасного ефективного методу визначення атразину дозволить швидко аналізувати значну кількість зразків для визначення залишків гербіциду. Польові досліди з вивчення залишків атразину у ґрунті та рослинах допомогли встановити закономірності їх накопичення. Ці дослідження мали теоретичні основи та практичні рішення щодо селекції сортів люцерни у різних регіонах сільськогосподарського виробництва.

Було проаналізовано стійкість 60 сортів люцерни до атразину з виявленням стійкого (SF8001 (SF)) та чутливого (Juneng 2 (J2)) методом визначення ґрунтової токсичності. Результати експерименту показали, що залишки атразину мали значну токсичну дію на люцерну, але різні сорти люцерни мали різну стійкість

до гербіциду. Було перевірено найбільш стійкий сорт люцерни SF з IC_{50} 14,8750 мг/кг і найбільш чутливий - J2 з IC_{50} 0,2424 мг/кг. Використання гідропонного методу для визначення інгібувальної дії атразину на різні за стійкістю сорти люцерни показало, що зі збільшенням гербіциду показники інгібування свіжої ваги SF і J2 поступово збільшувалися та були істотними. Результати вивчення токсичності гербіциду показали, що IC_{50} стійкого сорту (SF) становила 8,28, а IC_{50} чутливого (J2) - 0,29.

Вплив P450s (інгібітор малатіону) та GSTs (інгібітор NBD-CI) на ріст SF і J2 визначали за допомогою гідропонного методу. Результати показали, що найвища безпечна доза малатіону для SF і J2 становила 25,00 мг/кг, а найвища безпечна доза NBD-CI для SF і J2 - 0,25 мг/кг, без істотної різниці порівняно з контролем.

Було визначено вплив малатіону та NBD-CI на рівні резистентності SF та J2 з використанням гідропонного методу. Порівняно з використанням лише атразину, значення IC_{50} для стійкого та сприятливого сорту були значно знижені після комбінованої обробки малатіоном, із коефіцієнтами IC_{50} 3,02 і 2,21, для SF і J2 люцерни відповідно. Тому було припущено, що стійкість SF і J2 до атразину може бути пов'язана з P450s. Подібним чином, порівняно з використанням лише атразину, IC_{50} для стійкого та чутливого сортів значно знизились після комбінованого застосування NBD-CI. Значення IC_{50} для SF і J2 становили 1,08 і 1,21, відповідно. Таким чином, було припущено, що стійкість резистентного та чутливого сортів до атразину не може бути пов'язана з GSTs.

За вивчення впливу атразинового стресу на висоту рослини (PH), довжину кореня (RL), суху вагу пагона (SDW) і суху вагу кореня (RDW) різних за стійкістю сортів встановили такі закономірності: зі збільшенням концентрації внесення атразину ці показники зменшувались, різниця у пригніченні SF і J2 була значною, чутливий до атразину сорт пригнічувався сильніше за стійкий. Коли концентрація гербіциду становила 2,0 мг/кг, SF знижував PH, RL, SDW і RDW на 50,84%, 55,03%, 44,42% і 54,13%, відповідно, під впливом атразину. J2

зменшив ці показники на 81,84%, 81,73%, 92,31% і 85,00% відповідно. Результати показали, що стійкість SF і J2 до атразину пов'язана з фотосинтезом.

Вивчення впливу атразинового стресу на фотосинтетичні характеристики SF і J2 показало, що зі збільшенням концентрації атразину в ґрунті чиста швидкість фотосинтезу (Pn), продихова провідність (Gs) і швидкість транспірації (Tr) поступово знизились, тоді як міжклітинна концентрація вуглекислого газу (Ci) поступово збільшилась. При концентрації атразину 2,00 мг/кг Pn, Gs, Tr і Ci у сорта J2 зменшились на 83,42%, 84,15%, 91,57% і 46,69%, відповідно. При 0,5 мг/кг - Pn, Gs і Tr у сорту SF знизились на 34,03%, 17,90% і 27,59%, відповідно, тоді як Ci збільшився на 16,38% і продемонстрував значні відмінності порівняно з J2.

Визначення впливу атразинового стресу на параметри флуоресценції хлоропластів різних за стійкістю сортів показало, що зі збільшенням залишкової кількості атразину максимальний квантовий вихід (Fv/Fm), максимальна фотохімічна ефективність (Fv/Fo), фактична фотосинтетична ефективність (Y(II)), фотосинтетична швидкість транспорту електронів (ETR), і коефіцієнт фотохімічного гасіння флуоресценції (qP) зменшилися, тоді як коефіцієнт нефотохімічного гасіння (NPQ) мав протилежні зміни. У концентрації атразину 2,00 мг/кг показники Fv/Fm, Fv/Fo, Y (II), ETR і qP зменшилися відповідно на 11,76%, 76,90%, 80,00%, 63,12% і 46,44%, тоді як NPQ вірогідно збільшився на 37,64 % порівняно з контролем. Коли концентрація гербіциду становила 0,50 мг/кг, то Fv/Fm, Fv/Fo, Y (II), ETR і qP сорту SF зменшилися на 10,11%, 8,00%, 24,56%, 26,62% і 11,11% відповідно, тоді як NPQ збільшився на 19,72%, що значно відрізнялося від J2.

Вміст хлорофілу та MDA (малонового діальдегіду) у SF та J2 зазнали впливу після застосування атразину. Результати показали, що зі збільшенням днів застосування вміст хлорофілу *a* та хлорофілу *b* у SF залишався вищим, ніж у J2. На 10-ту добу вміст хлорофілу *a* в SF та J2 перевищував контроль у 1,35 та 4,53 рази, відповідно, а вміст хлорофілу *b* – у 3,4 та 1,06. Вміст MDA у SF

залишався нижчим, ніж у J2, із збільшенням кількості днів застосування. На третій день уміст MDA у J2 був у 4,19 разів вищим за контроль, і в 1,91 разів вищим за SF з дещо повільнішим темпом зростання через три дні. Стійкість SF і J2 до атразину була пов'язана з активністю захисних ферментних систем.

Оптимізували умови методу UPLC-MS/MS для визначення атразину в рослинах люцерни. Було оцінено відмінності в поглинанні та метаболізмі гербіциду між SF та J2 за допомогою цього методу. Результати показали, що залишкова кількість атразину в J2 завжди була вищою, ніж у SF, і була значна різниця. Стійкий сорт мав значно вищу швидкість метаболізму атразину, ніж чутливий. Поява резистентності до гербіциду у люцерни може бути пов’язана з підвищеним рівнем стійкості до поглинання та метаболічної здатності.

Порівняльний аналіз генів SF і J2, змінених під впливом атразинового стресу, з використанням технології секвенування транскриптомів, дозволив визначили 4032 диференціально експресовані гени у надземній частині між J2-T і SF-T, з яких 2233 були з підвищеною регуляцією, а 1799 з низькою. Результати аналізу GO та KEGG показали, що диференціально експресовані гени головним чином залучені до фотосинтетичного використання вуглецю та метаболізму, синтезу амінокислот та галактозилтрансферази UDP.

Визначення залишків атразину у ґрунті та рослинах впродовж двох вегетацій люцерни (2022-2024 рр.) показало загрозу для росту рослин. У ґрунті кількість атразину поступово зменшувалась, а потім стабілізувалась. Залишкова кількість у сортів SF і J2 спочатку збільшилась, а потім зменшилась.

Ключові слова: *люцерна, бобові, атразин, токсичність, засоби захисту, заходи захисту, шкідливість, залишки пестицидів, розвиток рослин, сходи, транскриптом, селекція, сорт, прогноз генотипу.*

LIST OF PUBLISHED WORKS ON THE TOPIC OF THE DISSERTATION

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1. **Zhu Y.**, Rozhkova T. Study on toxicological effects of herbicide atrazine on alf alfa seedlings. Bulletin of Sumy National Agrarian University. The Series: Agronomy and Biology. 2023. Vol. 53. Pp. 3–8. DOI: https://doi.org/10.32782/agrobio.2023.3.1
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5. **Zhu Y**., Rozhkova T., Zhu H. Common weed species in wheat fields in Henan province, China. Fundamental and applied problems of modern ecology and plant protection. Materials of the International Scientific and Practical Conference dedicated to the 100th anniversary of the birth of doctor of biological sciences, professor B.M. Lytvynov (Kharkov, October 21-22, 2021). Kharkiv: Ivanchenko I. Publishing House. P. 57-59.
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CONTENT

ABBREVIATIONS

SF8001: SF

Juneng 2: J2

UPLC-MS/MS: ultra-performance liquid chromatography-mass spectrometry

CK: without other treatment

P450s: Cytochrome P450 monooxygenases

GSTs: Glutathione-s-transferase

NBD-Cl: 4-chloro-7-nitrobenzaldehyde

IC_{50}: Lethal Concentration 50

GSH: Reduced glutathione

GTs: Ghicosyltransferases

ABC: ATP-Binding cassette

cDNA: Complementary DNA

ChII: chlorophII

DEGs: Differentially pressed genes

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

MS: Mass spectrometry

PSII: PhotosystemII

QRT-PCR: Quantitative real time-PCR

RNA-seq: RNA-sequencing

ATP: Adenosine Triphosphate

RSD: Relative standard deviation

TSR: Target site resistance

NTSR: Non-target site resistance

DNA: Deoxyribonucleic acid

PCR: Polymerase chain reaction

bp: Base pair

CYP450: Cytochrome P450

PH: Plant height

RL: Root length

SDW: shoot dry weight

RDW: root dry weight

Pn: net photosynthetic rate

GS: stomatal conductance

Tr: transpiration rate

Ci: intercellular carbon dioxide concentration

Fv/Fo: PSII maximum quantum yield

Fv/Fm: maximum photochemical efficiency

Y(II): actual photosynthetic efficiency

qP: PSII coefficient of photochemical fluorescence quenching

ETR: photosynthetic electron transport rate

NPQ: non-photochemical quenching coefficient

MFS: major facilitator superfamily

HXT2: Sweet sugar transporter

Ps: photosynthesis

FDR: False Discovery Rate

FPKM: Fragments per kilobase million

Ha: Hectare

KH_2PO_4: Potassium dihydrogen phosphate

LOQ: Limit of quantitation

LOD: Limit of detection

MDA: Malondialdehyde

Mg: Milligram

mL: Milliliter

min: Minute(s)

RISQ: Resistance In Season Quick

R^2: Correlation coefficient

ST: Sensitive treatment group

RT: Resistance treatment group

TSR: Target resistance

NTSR: Non-target resistance

GPX: peroxidase

TBA: thiobarbituric acid

PCA: Principal Component Analysis

HAAS: Henan Academy of Agricultural Sciences

PPPI: Plant Protection Pesticide Institute

XXYYB: Xinxiang Yuanyang Base

INTRODUCTION

Alfalfa is a perennial leguminous forage known as the "King of Forage" because of its high yield and good quality. It can be made into silage or hay for preservation and is an indispensable raw material for feeding the breeding industry (Graymore et al., 2001; Yu et al., 2017). However, because alfalfa cultivation consumes much water and continuous cropping is affected by autotoxic effects, it must be plowed after a certain number of years and rotated with other crops (Capel et al., 2001). As an essential food crop in China, planting corn with alfalfa to improve land resource utilization efficiency has been widely implemented. It has achieved higher yield, quality, and economic benefits. Atrazine is one of the most commonly used herbicides in corn and wheat fields (Cox, 2001; Cooper et al., 2007; Fan et al., 2014), its primary mechanism of action is to compete with plastoquinone for the binding site of the photosynthetic center on the thylakoid membrane, blocking the interaction between the electron acceptor protein and plastoquinone. The electron transport chain specifically interferes with photosystem II (PSII), reducing the efficiency of the CO_2 fixation process and thereby affecting the average growth and development of target weeds (Graymore et al., 2001; Héquet et al., 2001; Hayes, 2004).

Actuality of theme. Atrazine has a long persistence in the environment and remains in the soil for about a year. Many studies have shown that atrazine residues in crop rotations can affect the average alfalfa growth in the following crop. Therefore, atrazine toxicity is a common problem in corn-alfalfa cropping models. At present, relevant research mainly focuses on the poisoning mechanism of atrazine on alfalfa. However, there is no research on the differential resistance mechanism of different alfalfa varieties to atrazine stress. Therefore, this experiment provides a theoretical basis for the breeding of atrazine-resistant varieties of alfalfa and the study of its internal mechanisms.

Connection of work with scientific programs, plans, and themes. This study was conducted within the framework of a specific scientific topic, in collaboration with Sumy National Agricultural University and the Henan Provincial Key Science and

Technology Project "Research and application of herbicide soil residue pest control technology" (grant number 221111112300).

The purpose and objectives of the study. The purpose of the study is to determine the toxic effect of atrazine on plants and mechanisms of alfalfa resistance to this herbicide.

To achieve this goal, the following tasks were completed:

- to determine the resistance level of 60 alfalfa varieties to atrazine;
- to verify whether the P450s inhibitor malathion and GSTs inhibitor NBD-CI were related to the resistance of SF and J2 to atrazine;
- to determine the differences in physiological and biochemical indicators as well as photosynthetic characteristics between SF and J2 after application of atrazine;
- to use UPLC-MS/MS to detect the differences in absorption and metabolism of atrazine in the shoot and root parts of SF and J2;
- to analyze the reasons for the differences between SF and J2 genes from a molecular perspective;
- to verify the differences in field residues between SF and J2 alfalfa.

The object of study. Improving alfalfa breeding for atrazine resistance.

Subject of study. Alfalfa varieties, interaction of alfalfa plants with atrazine, atrazine toxicity, atrazine residues, mechanisms of alfalfa resistance to atrazine, transcriptome, expressed genes

Research methods. General scientific methods: analysis, induction, deduction, synthesis, and field methods. Phenological observations of alfalfa variety collection and biometric parameters of plant growth and development, especially plant height, fresh weight, and control effect analysis statistical methods - to summarize and determine the basis for specific experimental results (variations, correlations, dispersion, clustering, facts) reliability.

The scientific novelty of the results obtained lies in the detailed study of the harmful effects of atrazine on alfalfa, as well as in the disclosure of the mechanisms of plant resistance to the atrazine.

On the basis of analytical and experimental research, the work for the first time:

1. The most atrazine-resistant alfalfa variety - SF and the most favorable -J2 were identified.

2. The P450s inhibitor malathion was associated with resistance to SF and J2, while the GSTs inhibitor NBD-CI was not.

3. Atrazine had negative effects on the growth, physiological and biochemical indicators, and photosynthesis of SF and J2, with significant differences in their effects.

4. The ultra-high performance liquid chromatography method was used to detect significant differences in the absorption and metabolism of atrazine in the aboveground and root parts of SF and J2.

5. A comparative analysis of transcriptomes of resistant and sensitive alfalfa varieties under atrazine stress was carried out and key genes SF and J2, which were responsible for photosynthesis and carbon metabolism, synthesis of amino acids and galactosyltransferase, were identified.

6. The peculiarities of atrazine accumulation in plants of SF and J2 varieties under field conditions were shown.

The obtained data on the harmfulness and mechanisms of atrazine resistance of different alfalfa varieties will further provide a theoretical basis for breeding varieties resistant to this herbicide. In field experiments, it was confirmed that the absorption of atrazine by resistant and susceptible alfalfa shows an increasing trend, and the degradation of atrazine by the soil shows a decreasing trend. Thus, this provides a theoretical basis for the scientific and rational use of atrazine.

The practical significance of the results. Based on the research results, alfalfa varieties resistant to atrazine were planted, and the toxic relationship between atrazine residues and alfalfa was clarified. The yield and quality of alfalfa were improved, and the soil pollution environment was improved. At the same time, the introduction of excellent varieties, the knockout of genes sensitive to atrazine, and continuous improvement of planting techniques have increased the yield and quality of alfalfa, reduced atrazine environmental pollution, and contributed to the sustainable

development of agriculture. It has also played an important role in improving the ecological environment and alleviating the shortage of high-quality domestic forage. This is being studied and improved in the scientific project of the Henan Academy of Agricultural Sciences (Henan, China).

The results obtained are included in the training programs for junior bachelors, bachelors and masters in specialty 202 "Plant Protection and Quarantine" of Sumy National Agrarian University.

The applicant's contribution is to plan and conduct research, summarize scientific data from references (literature) on the dissertation topic, analyze experimental data, format conclusions and recommendations for selection, and write scientific papers. Scientific articles have been published both independently and in co-authorship.

Approbation of dissertation results. The results of the research were published and discussed at "International Scientific and Practical Conference dedicated to the 100th anniversary of the birth of Doctor of Biological Sciences, professor B.M. Lytvynov" (Kharkiv, 2021), International Scientific and Practical Conference dedicated to the anniversary dates of the birth of outstanding scientists-phytopathologists, Doctors of Biological Sciences, Professors V. K. Panteleyev and M. M. Rodigin (Kharkiv, 2022), IV All-Ukrainian Scientific and Practical Conference (Zhytomyr, 2024).

Publications. Based on the research results, three articles were published in professional journals, one articles were published in Scopus (Q3), and three papers were exhibited at conferences as the first author.

The structure and scope of the dissertation. The dissertation structure contains an annotation, a list of abbreviations, an introduction, seven chapters, conclusions, a list of references, and appendixes. In this dissertation, it includes twenty tables and twelve-five figures.

CHAPTER 1

STUDY ON MECHANISM OF RESISTANCE OF ALFALFA TO ATRAZINE (LITERATURE REVIEW)

Current status of alfalfa development

Alfalfa (*Medicago sativa L.*) is one of the essential perennial leguminous grasses (Lazorko et al., 2009; Pathak & Dikshit, 2011), it has a high yield, good quality, and intense stress resistance and is known as the "King of Forage," it can not only be used as an animal grazing ground to establish artificial grassland. However, it can also be used to make silage or hay for preservation, playing an essential role in developing agriculture and animal husbandry. After nearly ten years of development in China's industry, the current commercial grass planting area has increased to about 9 million acres, but it still cannot meet the dairy industry's demand for it (Zhu & Rozhkova, 2024). In 2022, hay imports from other countries reached 1.79 million tons, and the unit price was close to soybeans, far exceeding wheat and corn (Singh et al., 2018). As a highly adaptable feed crop, its well-developed root system can utilize water in deep soil and improve soil fertility through nitrogen fixation, improve the local ecological environment, and reduce soil erosion (Singh et al., 2018). However, its vigorous growth, well-developed root system, and high yield also cause its cultivation to consume much water, leaving the bed for a long time will not only reduce the yield due to its reasons but also cause the soil layer to dry out due to the large consumption of soil water, forming deep dry patches. The soil layer affects the average growth and development of it. Therefore, it is usually plowed after 4-5 years of planting (Solomon et al., 2008; Udiković-Kolić et al., 2012). However, continuous cropping seriously affected due to its autotoxic effect. Therefore, other crops were usually planted for 1-2 years before being rotated. With the further development of animal husbandry, improving land resource utilization efficiency to increase forage production has become a significant challenge (Komang et al., 2002). Corn and wheat are some of the food crops with the largest planting area in the world and are also the most critical energy crops, one is to rotate crops sown in summer with it in autumn after the harvest of corn, which is

currently a widely used planting model, this planting model of perennial leguminous crops combined with annual food crops has been widely promoted around the world, and yield, quality and economic benefits have been significantly improved (He et al., 2019). The alfalfa-corn planting model can not only provide high-quality feed for livestock, which is conducive to the development of a sustainable livestock system but can also use it and rhizobia to fix nitrogen symbiotically to provide nutrients for the growth of corn (Ralston-Hooper et al., 2009). However, atrazine is one of the most commonly used herbicides in corn fields. Whether it is the soil residue in the corn and its rotation model or the selection of herbicides in the intercropping model, its Harm is an issue that needs to be solved urgently (Pratt et al., 2003).

1.1. Overview of atrazine

1.1.1. Physical and chemical properties and mechanism of action of atrazine

Atrazine is a triazine herbicide, its chemical name is 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, its molecular formula is $C_8H_{14}ClN_5$, its molecular weight is 215.69, it is a white powder with a density of 1.2 g/mL (20°C) and a melting point of 173-175℃, the boiling point is 200℃, vapor pressure is 4.0×10^{-5} Pa (20℃), hardly soluble in water, easily soluble in organic solvents, solubility in water is only 33 mg/L, chloroform is 28 g/L, acetone it is 31 g/L, ethyl acetate is 24 g/L, and methanol is 15 g/L. Atrazine is relativelystable under weakly acidic or weakly alkaline conditions. It can be degradedbyhigh temperature, strong alkali, or strong acid. The degradation products mainly include deethylatrazine (DEA) and deisopropylatrazine (DEA). Deisopropylatrazine, DIA) and hydroxyatrazine (Hydroxyatra**-**zine, HYA), etc. H. Geising andE. Knussli of Switzerland in 1957 discovered the herbicidal properties of atrazine. It was developed and produced by Geigy of Switzerland in 1958 and laterdeveloped into one of the world's most produced herbicides (Mudhoo & Garg, 2011).

Atrazine is a systemic selective pre-emergence and post-emergence herbicide that can control a variety of annual grass and broadleaf weeds and is widely used in corn, sorghum, sugarcane, etc. (Bianchi et al., 2006). Atrazine is mainly absorbed by plant

roots and less by stems and leaves. After application, it is transmitted to the meristem tissue and leaf parts of the plant, destroying chlorophyll and hindering the production of plant carbohydrates (Jablonowski et al., 2011). Its mechanism of action is mainly to inhibit weeds by interfering with photosystem II, it is absorbed through the roots of weeds and conducted upward, competing with plastoquinone for the D1 protein binding site in the thylakoid membrane, thereby inhibiting photosystem II (PSII), blocks photosynthesis, damages chlorophyll synthesis and cell membranes, and ultimately leads to cell death (Organization, 2003; Park et al., 2003; Zhang et al., 2014).

1.1.2. Usage of atrazine

Atrazine is a universal, broad-spectrum, long-acting herbicide widely used in farmland to control broadleaf weeds and grass weeds, it has selective systemic conductivity and can be used in pre-emergence and seedling applications. Atrazine has high water solubility and can penetrate deep into the surface through rainwater leaching, so it also has herbicidal activity against some deep-rooted weeds (Zhu & Rozhkova, 2024). Due to its excellent weeding effect and low price, it is widely used worldwide to remove broadleaf weeds, grass weeds, and certain perennial weeds in corn, sorghum, and sugarcane fields. In addition, it is also widely used in tea plantations, orchards, and woodlands, as well as in non-agricultural aspects such as road maintenance (Hayes et al., 2011). Atrazine has been used as a chemical herbicide worldwide for more than 50 years, and the global annual use of atrazine is 70,000 to 90,000 tons. Since the 1980s, China has been the leading producer and user of atrazine, and the annual use of atrazine in China has reached tens of thousands of tons (Cooper et al., 2000; Chan et al., 2004).

1.1.3. Toxic effects of atrazine

The negative impact of atrazine on ecosystems is not only reflected in crop yield and quality but also the quality of environmental ecosystems, the harm of atrazine to the ecological environment is global (Moreno et al., 2007). Although the solubility of atrazine in water is extremely low, it can be transferred globally through precipitation, leaching, surface runoff, etc. Scholars from various countries have conducted extensive research on the toxicity of atrazine, involving many fields such as biology, physiology,

environmental science, and toxicology (Hayes et al., 2002). Atrazine pollution is becoming increasingly severe, which has caused public and academic circles to pay attention to atrazine pollution.

1.1.3.1. Effects of atrazine on water quality and aquatic life

In the theoretical connotation and basic principles of sustainable water environment management, more emphasis is placed on the effective treatment of industrial wastewater and domestic sewage (Barr et al., 2007). At the same time, multi-data integration and multi-disciplinary intersection jointly promote the sustainable development of ecology, which has become a management trend. However, atrazine and its metabolites were found in rivers, lakes, and seas in many areas of my country. Previous researchers tested the water of Guanting Reservoir and found trace amounts of the endocrine disruptor atrazine (Jablonowski et al., 2010). Although it did not exceed my country's current surface water standards, this area is an essential source of drinking water for Beijing, if consumed for a long time, it will also damage the human immune system and endocrine system, causing potential harm to the body's functions.

The widespread use of herbicides also poses severe threats to aquatic ecosystems. Previous researchers found that the photosynthesis of a type of algae, Scenedesmus obliquus, was significantly reduced in an atrazine environment (Chingombe et al., 2006). Although the concentration of atrazine did not affect algae growth, it increased the risk of herbivores' predation in the entire water area, damaging the entire community structure. Previous researchers used the sealed triangular bottle mouth method to study the anti-hypoxia ability of crucian carp under atrazine stress (Santos & Martinez, 2012). The results found that when the concentration of atrazine is high, it will threaten the anti-hypoxia ability of crucian carp, indicating that the water body atrazine may cause a decrease in the activity of antioxidant enzymes in crucian carp, showing a state of sustained low activity. Previous researchers used qPCR technology and transmission electron microscopy to study the changes in the antioxidant enzyme system of carp under different pesticides (atrazine and chlorpyrifos) and at different concentrations and the molecular and physiological mechanisms of detoxifying

herbicides, proving that iNOS (induced type nitric oxide synthase) participates in the detoxification process of atrazine and chlorpyrifos, and reveals for the first time the oxidative stress-induced autophagy of carp under atrazine and chlorpyrifos stress (Wackett et al., 2002). In addition, researchers also found that not only does the parent atrazine affect and threaten the growth and physiology of fish, but its metabolites desethylatrazine (DE) and desisopropylatrazine (DIP) and diaminochlorotriazine (DACT) also have adverse effects on fish growth. Their metabolites are even more toxic than the parent atrazine (Tappe et al., 2002).

1.1.3.2. Effects of atrazine on soil and plants

The half-life of atrazine in the soil is very long and usually remains for several days to several months (Kniewald et al., 2000; Shipitalo et al., 2003; Kong et al., 2016). The half-life of atrazine is greatly affected by environmental factors, mainly the physical and chemical properties of the soil, previous farming history, crop characteristics, and the influence of indigenous microbial species (Rohr & McCoy, 2010). Commercialized atrazine has been banned in Germany, Italy, Australia, and the Netherlands. However, atrazine is still in production and widely used in China, which causes increasingly serious harm to soil and the environment (Wang et al., 2005). China's major atrazine pollution accidents caused substantial economic losses and severe damage to the ecological environment, which cannot be fully restored within a few years or even decades, these "historical issues" have caused us to think deeply (Abdelhafid et al., 2000). In order to understand the sensitivity of different plants to atrazine, many scholars have studied the toxicological characteristics of plants in response to atrazine in order to identify the main dominant weed species in farmland and explore strategies to optimize the use of atrazine in farmland. Provide a theoretical basis for the practical application of farmland atrazine and research on the control of resistant plants (Seffernick et al., 2000; Accinelli et al., 2001).

1.1.3.3. Harm of atrazine to the human body

Atrazine has a global impact on the entire ecological environment, it can be transferred to other areas through water runoff, leaching, etc., and atrazine in the soil

can be released through water volatilization and plant respiration, diffuses into the atmosphere, where it is recycled and eventually absorbed by the human body (Diana et al., 2000). Taking workers engaged in the production and maintenance of commercial atrazine as an example, researchers analyzed atrazine and its metabolites in their urine. They found that after 12 hours of no longer being exposed to atrazine, the levels of atrazine in their urine were still high doses of pesticide residues (Friedmann, 2002; Ventura et al., 2002; Bolle et al., 2004). Although the degradation mechanism of atrazine in human liver micro-mitochondria and cytochrome P450 monooxygenases (P450s) has been clarified, the harm of atrazine as an endocrine disruptor to the human body cannot be ignored. The latest research report also pointed out that atrazine in the environment can even increase the risk of premature babies and malformed babies. The problem of atrazine contamination is imminent, and reducing the use and remediating pesticides are the keys to current research hotspots (Alla et al., 2006).

1.2. Mechanism of resistance

The resistance mechanisms of weeds to photosystem II inhibitor herbicides can be mainly divided into target-site-based resistance (TSR) and non-target-site-based resistance (NTSR) (Kreslavski et al., 2007; Qi et al., 2021). The cause of TSR may be the mutation of the psbA gene on the D1 protein, which leads to changes in the membrane protein structure, which in turn reduces the herbicide sensitivity corresponding to the target site, NTSR mainly involves the absorption of herbicides in weeds (Kreslavski et al., 2013b; Yang et al., 2016). The entire process of herbicide, conduction, and metabolism, such as reduction in osmotic absorption, changes in transport rate or direction, and enhanced metabolic capacity, can lead to a reduction in the number of herbicides reaching the target site, leading to the occurrence of resistance (Puangbut et al., 2022).

NTSR and TSR are the main resistance mechanisms of weeds to PSII inhibitor herbicides. NTSR involves weeds' absorption, conduction, and metabolism of PSII inhibitors; TSR is mainly based on mutation of the psbA gene site, resulting in

decreased sensitivity of the target site (Guo et al., 2024).

1.2.1. Target resistance

One of the main mechanisms by which weeds develop resistance is the mutation of herbicide target genes, which mostly occur in conserved regions of dominant or semi-dominant genes (Colom et al., 2003; Irmak et al., 2008). Nowadays, there are more and more reports of target gene mutations causing weed resistance (Calzadilla et al., 2022). It has now been confirmed that the target resistance of weeds to PSII inhibitors has been studied, and it concluded that the psbA gene of the D-1 protein in the reaction center is mutated, resulting in a decrease in the affinity of PSII inhibitor herbicides to the D-1 protein, thereby reducing herbicides agent activity. D1 protein is encoded by the psbA gene in higher plants, algae, and cyanobacteria (Basma et al., 2011). These sequences are highly conserved, and the gene is maternally inherited in plants. In higher plants, the psbA gene exists in a single copy. Studying the impact of single amino acid substitutions in psbA is of great significance for elucidating this protein's molecular mechanism of action (Sukhov et al., 2014; Silveira & Carvalho, 2016). Target resistance often causes weeds to develop higher resistance levels to PSII inhibitors. In 1988, Poa- annua was resistant to atrazine due to a mutation in the psbA gene of the D1 protein; it was also the first time that the Ser-264-Gly mutation in the psbA gene was reported, which resulted in a decrease in the binding ability of the herbicide to thylakoid, and thus develop resistance (Mommer et al., 2005; Kumudini et al., 2010; Kreslavski et al., 2013a). Researcher has found that the Ser-264 Gly mutation is present in the psbA gene of the D-1 protein in most herbicide simazine-resistant lettuce biotypes (Verma et al., 2021). Previous studies compared the differences between different Solanum nigrum populations at the DNA level using DNA (RAPD) detection technology. The results showed that 7 of the 25 populations collected from Poland, France, and the United Kingdom showed high tolerance to trinitrogen. Benzene herbicides have developed resistance, and target resistance testing found that there is a mutation in the 264th amino acid of the psbA gene, from serine to glycine (Sun et al., 2022); someone also reported the discovery of amaranth biotypes

with a Ser-264-Gly mutation in the psbA gene, it is precisely because of this mutation that the amaranth becomes resistant to triazobenzene herbicides (Francesconi & Balestra, 2020). In most cases, the Ser-264-Gly mutation in the D1 protein is the main cause of weed biotype resistance to triazobenzene herbicides (Choat et al., 2011). Ser-264-Gly can cause weeds to develop a high resistance level to triazobenzene herbicides (resistance multiple >50). At the same time, it does not produce resistance or different levels of resistance to substituted urea herbicides (Hu et al., 2022). In 2017, it was reported that the urea herbicide Liguron developed high levels of resistance, both Ser-264-Gly and Ser-264-Thr mutations inhibited the interference of PSII inhibitory herbicides on electron transfer in photosynthesis (Su et al., 2017), research has also shown that in many organisms, Phe-211-Ser, Gly-256-Asp, Leu-275-Phe, both can cause herbicides to develop resistance, the Ser-264 mutation mainly leads to weed resistance to triazolone, uracil, and other PSII inhibitory herbicides, in addition to Phe265, amino acid binding sites such as Phe-255, Ala-251, and His-215 can affect the binding of herbicides to D-1 protein (Li et al., 2023). In 2017, Souza found that the resistance of weeds to PSII inhibitor herbicides was also influenced by the presence of a Ser-268-Pro mutation in the psbA gene through sequencing (Souza et al., 2017). PCR technology was used to selectively amplify the herbicide binding regions of 20 chloroplast psbA genes resistant to glyphosate and diclofenac in poa pratensis. Sequence analysis showed that this fragment had a Val-219- Ile mutation in the D1 protein encoded by the psbA gene. Other non-triazine-based PSII inhibitors may exhibit different resistance outcomes due to their different D1 protein binding sites and chemical structures (Li et al., 2017). Previously found that the valine at position 219 was encoded by the psbA gene of kochia chinensis that is resistant to diuron, and betatron was replaced by isoleucine, this mutation caused Kochia chinensis to be resistant to diuron, cross-resistance was induced to trituration, submarine, linuron, and bromoxynil, with high resistance to diuron and moderate resistance to magazine and atrazine (Chen et al., 2015). Previous found that the Val-219-Ile mutation in the psbA gene can cause drug-resistant biotypes to become resistant to diuron, imazam, and

bromoxynil but not to bendasone (Mitra & Baldwin, 2008). Previous identified eight psbA gene loci related to resistance to PSII-inhibiting herbicides in resistant wild carrots, and two mutations, Ser-264-Gly and Phe-274-Val, were detected, among which Phe-274-Val have never been found in resistant biotypes before. Further research found that Phe-274-Val can cause wild carrots to respond simultaneously to three PSII inhibitors of different structures, atrazine and mesotrione. and diuron conferred moderate levels of resistance, whereas bromoxynil was susceptible. Target resistance often causes weeds to develop higher resistance levels to PSII inhibitors. In particular, the Ser-264-Gly mutation causes the highest resistance level and is currently the most common mutation site (El-Lithy et al., 2005).

1.2.2. Non-target resistance

The non-target resistance mechanism reduces the concentration of herbicide that interacts with the target site, including reducing herbicide uptake and transport, enhanced herbicide metabolism, and herbicide sequestration (Khan et al., 2023). Previous summarized the relevant factors affecting herbicide leaf absorption and root absorption, the differences in herbicide absorption between species are mainly attributed to differences in root morphology and differences in the physical and chemical properties of the leaf surface, these differences affect the absorption of herbicide solutions on the leaves, the retention time and the efficiency of herbicide penetrating the epidermis, such as hairy leaves with fluff are easier to retain mist droplets than smooth leaves, thus promoting the absorption of the herbicide (Patakas et al., 2003; Feng et al., 2014; Sun et al., 2023).

Reduced herbicide transport was first demonstrated in glyphosate-resistant ryegrass populations, where weeds develop resistance by retaining the herbicide on the applied leaves, preventing its transport to the growing point. In sensitive ryegrass populations, glyphosate mainly accumulates in the roots. In contrast, in resistant populations, glyphosate accumulates on the edges of leaves, with less pesticide transferred to the meristem, thereby reducing herbicide efficacy. In addition, in the glyphosate-resistant P. flora population, differences in glyphosate transport are responsible for resistance

(Nogueira et al., 2020).

Metabolic resistance is currently the most common non-target resistance mechanism, which is caused by P450s and GSTs in plants (Borggreve et al., 2003; Moores et al., 2009; Panini et al., 2016). It is caused by the action of enzymes and glycosyl transferases, which metabolize the herbicides into non-toxic or low-toxic secondary metabolites. Herbicide detoxification metabolism generally includes three stages, the first stage is usually the redox effect mediated by P450s enzymes; the second stage is the conjugation of GSTs and GT-catalyzed metabolites, and some herbicides can also be inactivated by direct reaction with GSH, the third stage is to transfer metabolites out through vacuoles or transporters (Hatzios & Burgos, 2004; Zhang & Yang, 2021). Studies have shown that overexpression of CYP81A12 and CYP81A21 genes leads to resistance of E. phyllopogon to bifenthrin and bensulfuron-methyl (Riar et al., 2015). The researcher reported that the resistance of barnyard grass to oxazoline is related to the enhanced activity of P450s enzymes (Chin & Management, 2001), a researcher reported that enhanced P450s and GSTs enzyme activities caused barnyard grass resistance to penoxsulam (Chen et al., 2016). The synergistic effect of different enzyme systems metabolizes or blocks the herbicide before reaching the target site, leading to the development of resistance (Yasuor et al., 2009; Yan et al., 2019; Feng et al., 2022).

The enhanced metabolism of alfalfa causes barnyard grass to become resistant to one or more herbicides with different action sites, and there is a risk of developing resistance to new herbicides, making the management of barnyard grass more challenging and having a significant impact on agricultural production. (Chin & Management, 2001; Wang et al., 2019).

1.3. Overview of research on abiotic stress by alfalfa

As sessile organisms, plants cannot escape from the place where they grow, so they often suffer from environmental stress that is not conducive to plant growth and development, mainly divided into biotic stress and abiotic stress (Ashraf et al., 2018; Mareri et al., 2022). As environmental pollution problems become more and more

severe, extreme weather becomes more and more frequent, and heavy metal and chemical pollution in the plant growth environment become more and more serious, how to improve the resistance of plants to abiotic stress is a crucial issue to ensure crop yields (De et al., 2014).

More than 70% of the world's freshwater resources were used for agricultural production, and with climate change, the growth of crops in arid and semi-arid areas is increasingly suppressed due to lack of available water (Farooq et al., 2012; Seleiman et al., 2021; Yang et al., 2021). Kang and others treated alfalfa through the natural drought method, the results showed that the plant height, yield, and photosynthesis-related indicators of alfalfa were significantly reduced (Kang et al., 2011), the results of the greenhouse sand cultivation research conducted by researchers showed that the plant morphology and root system development of alfalfa is different under different water potential gradients (Liu et al., 2018). Researchers who researched alfalfa with different root types under drought stress showed that the root system morphology of alfalfa with various root types will be affected by drought stress (Zhang et al., 2019). The above experimental results proved that the plant morphology of alfalfa will undergo significant changes under drought stress, and the growth of leaves under drought stress is affected and is often accompanied by stomata closing and other phenomena, affecting expected light effects and reducing biological activity. Drought stress can also cause specific oxidative damage to alfalfa and activate the plant's antioxidant system (Hamidi et al., 2010). Previous researchers compared the physiological indicators of two alfalfa varieties in response to drought stress. The results showed that the MDA in the two varieties of alfalfa plants The activity of SOD, proline, and other antioxidant substances increased significantly. These test results illustrate that within the antioxidant capacity of alfalfa, the plant will respond to drought stress by activating its defense mechanism. Thermal damage to alfalfa caused by high temperatures in summer is an important factor restricting the high yield of alfalfa in my country. High-temperature stress can cause plant growth to slow down and affect its average physiological and biochemical function balance (Diatta et al.,

2021). Predecessors' research also found that high temperatures can cause increases in electrical conductivity and cell membrane permeability.

As a perennial pasture, alfalfa often suffers severe freezing damage in winter due to sowing date issues or climate changes, which limits the productivity and survival rate of alfalfa in early spring and late autumn. Low-temperature stress problems, especially the low-temperature problem after sowing in the first year of autumn, are severe problems in production. Enormous challenges (Liu et al., 2022) and low temperatures will have a severe impact on plant photosynthesis, including PSII efficiency, ATP synthesis, and stomatal conductance (Castonguay et al., 2006; Mo et al., 2011; Liu et al., 2019). Researchers used alfalfa as a material to study its photosynthetic performance under refrigeration treatment (Wang et al., 2024); the results showed that chlorophyll fluorescence parameters were affected by low-temperature stress, the experiments conducted by Jiang et al. on alfalfa seedlings and Klos and Brummer on the low-temperature response mechanism of alfalfa at different growth stages, as well as the results of Zhu evaluation of low-temperature cold resistance of four alfalfa varieties, all demonstrate that these osmotic regulatory substances are related to the cold resistance of alfalfa (Klos & Brummer, 2000; Zhu et al., 2021; Jiang et al., 2023). Heavy metal stress is different from other abiotic stress methods. It is mainly caused by human industrial development and the production of various reagents or products. It has a wide range of pollution, many types, and significant differences, and is difficult to degrade through the original ecological cycle of nature, when heavy metals reach toxic levels, they will affect the average growth and development of plants and may also induce DNA mutations, root growth inhibition, necrosis, leaf discoloration, and other symptoms (Tchounwou et al., 2012; Ali et al., 2019).

Transcriptomics is a discipline that studies the transcription and regulation of genes; it can compare, screen, and analyze the differential expression of plants under various stress conditions, thereby understanding the relationships between gene expression, stress injury mechanisms, stress response mechanisms, and so on (Desikan et al., 2001; Kreps et al., 2002; Girgenti et al., 2021). At present, there is a large amount of research

on the differential expression of genes in plants under abiotic stress, such as low-temperature treatment of wheat young ears, salt alkali stress of wheat seedlings and roots, transcriptome analysis of maize seedling roots under alkaline stress, high-temperature treatment of maize leaves, and transcriptome analysis of soybean roots under iron deficiency stress and heavy metal Cd stress (Mahfoozi et al., 2001; Ben et al., 2008; Yang et al., 2008; Zhao et al., 2020). Transcriptomics also plays a vital role in exploring the stress resistance mechanism of alfalfa (Lei et al., 2018). Helaoui studies have shown significant upregulation of Prx1 and GSTs genes in stems and roots under nickel (Ni) stress, the gene expression of pcs was significantly enhanced at different nickel concentrations, indicating their essential role in nickel detoxification in alfalfa (Helaoui et al., 2020). Through transcriptome and metabolomics analysis, Chen found that amino acid metabolism and nicotinamide metabolism pathways play essential roles in graphene tolerance in alfalfa (Chen et al., 2021). Zeng identified that β-amylase, ethylene responsive factor (ERF), calcium-like phosphatase B (CBL) interacting protein kinases (CIPKs), glutathione peroxidase (GPX), and GST-related genes identified by comparative transcriptomics might play a vital role in the waterlogging tolerance of alfalfa. The above studies proved that transcriptomic analysis helped explore the molecular mechanism of alfalfa in response to atrazine stress.

1.4. Degradation and transformation of atrazine

Atrazine has a stable structure and slow degradation rate and is widely distributed in the environment. Its degradation is usually divided into biological degradation and non-biodegradation. The degradation process usually involves dealkylation, alkyl oxidation, dechlorination, and hydroxylation. chemical reaction (Bianchi et al., 2006; Siripattanakul et al., 2009; Udiković-Kolić et al., 2012). At the same time, moisture content, temperature, and pH will all affect the degradation rate of atrazine (Topp et al., 2000; Héquet et al., 2001; Komang et al., 2002).

1.4.1. Abiotic degradation

The non-biological degradation of atrazine in the environment is chemical

degradation, which mainly includes photodegradation, oxidation reaction, reduction reaction, and electrochemical oxidation (Ghosh & Philip, 2004; Hou et al., 2017). In the photodegradation test, atrazine in aqueous solution can rapidly decompose under 254 nm uv radiation. Through UPLC-MS/MS technology, many intermediates were separated and identified, it was found that direct photolysis mainly proceeds through the following seven pathways. Chloride removal Alkylation, dechlorination and hydroxylation, alkyl oxidation, dechlorination and hydrogenation, dechlorination and dealkylation, deamination and hydroxylation and olefination (Hong et al., 2019; Mathon et al., 2019). In addition, Hu proposed that microwave induction can degrade atrazine adsorbed in the micropores of mineral adsorbents. This degradation process is better than photochemistry in treating atrazine in polluted water, atrazine degrades very quickly under microwave irradiation, and this method avoids secondary contamination (Hu et al., 2012).

1.4.2. Biodegradation

Many studies have shown that atrazine degradation is mainly biodegradation (Govantes et al., 2009; Udiković et al., 2012; Bhatt et al., 2022). Zhu and Philip believed that biodegradation is the only way to completely mineralize atrazine and proposed the biodegradation pathway of atrazine (Zhu et al., 2019), many researchers have isolated and purified atrazine-degrading bacteria and used them for the remediation and treatment of atrazine-contaminated soil and atrazine-containing wastewater. Since 1982, atrazine-degrading strains have been isolated from multiple genus rhodococcus, nocardia, agrobacterium, and acinetobacter bacteria. The enzymatic reaction is the essential process of microbial degradation of atrazine (Rousseaux et al., 2001; Topp & Soils, 2001; Cai et al., 2003). Under the action of microorganisms, parent atrazine forms hydroxylated atrazine (HA), desmethyl atrazine (DEA), diisopropyl atrazine (DIA), etc., and atrazine hydroxylation metabolites are non-toxic to plants and vertebrates. Desethylatrazine and desisopropylatrazine are still toxic but less toxic than the parent. Souza and researchers found that hydroxy atrazine is degraded by pseudomonas through the cloning and partial characterization of a gene

(_p_MD4) of the genus Pseudomonas, which is crucial in the dechlorination process of atrazine (Barr et al., 2007; LeBlanc & Sleno, 2011).

Conclusions to chapter 1

Through the literature analysis, it is necessary to study the mechanism of alfalfa resistance to atrazine. Henan Province, China is my country's central herbivorous animal husbandry province. The number of dairy cows and mutton sheep in stock ranks among the top in the country, and the number of beef cattle in stock and slaughtered has ranked first in China for many years. Alfalfa is the foundation of animal husbandry development. Therefore, vigorously developing the alfalfa industry is the key to continuously promoting the healthy development of the alfalfa industry in Henan Province, China. However, the planting area in Henan Province, China, spans a wide range with significant environmental differences. Atrazine is a commonly used herbicide in corn fields, and corn-alfalfa rotation is a standard rotation planting pattern in the world. Atrazine residues will cause varying degrees of Harm to it. Therefore, screening alfalfa germplasm resources for atrazine-resistant alfalfa is significant for breeding new atrazine-resistant alfalfa varieties.

In recent years, in order to implement the national strategy of ecological protection and high-quality development of the Yellow River Basin in Henan province, China, and adjust the structure of breeding and farming, the Henan provincial government of China has launched the construction of a high-quality grass belt in the Yellow River beach area to create a "million-acre" high-quality forage base. Among them, in the early stage of constructing the nearly "100,000-acre" alfalfa base, one-third of the seedlings were harmed by the residual atrazine of the previous corn crop, resulting in nearly "10,000 acres" of reseeding. Therefore, reducing the harm of residual atrazine in the soil to alfalfa production has become an important issue facing the large-scale production of alfalfa. Therefore, the screening test of alfalfa for atrazine is the basis for crop genetic improvement. Varieties with inconsistent genetic bases have different tolerance to herbicides. Screening alfalfa varieties resistant to atrazine is an essential

direction for discovering alfalfa atrazine-tolerant genes and cultivating new alfalfa-tolerant varieties.

At present, relevant research mainly focuses on the toxic mechanism of atrazine on alfalfa, which can cause toxicity to various subsequent crops. We found through indoor experiments that there were differences in the sensitivity of 60 alfalfa varieties to atrazine, only when their resistance mechanisms were determined can they be better applied in agricultural production. Selecting the most resistant and sensitive alfalfa varieties for the study of resistance mechanisms, the reaction catalyzed by P450s inhibitor malathion and GSTs inhibitor NBD-CI is the initial step in atrazine metabolism, and whether they play a metabolic role in the formation of alfalfa resistance to atrazine is examined. Atrazine is a photosynthetic herbicide, and chlorophyll is an important pigment in photosynthesis, the MDA content indicated the degree of damage to plants and reflect the physiological and biochemical indicators, photosynthetic characteristics, and chlorophyll fluorescence parameters of two alfalfa varieties under atrazine stress, the surface also reflected the significant differences between the two plants. Transcriptome sequencing revealed genes involved in the differences between two varieties, which deeply explored the true reasons for the differences between the two alfalfa varieties.

Based on the results of the fourth chapter, 2 Ukrainian Journal and 1 Conference Papers were published (Zhu & Rozhkova, 2023; Zhu & Rozhkova, 2024; Zhu et al., 2021).

CHAPTER 2

MATERIALS AND METHODS OF RESEARCH

Materials:

60 alfalfa varieties: XL, YK, J2, 601, 801, FL, HG, 9720s, HX22-80, HX22-69, HX22-77, LYL, TM, J995, J201, HS, SD10, ZM3, WL168H, YS, 8421s, SF, J301, JNDDL, HX22-64, HX22-65, HX22-62, HX22-78, HX22-56, 5020, SD10-2, DT, J801, YST, JG, WS, LDN, J806, LD, WL366, WL343, GN5, AEGJ, WL440, SDL, YX, 4030, J405, HX22-66, HX22-58, SH, WL363, J5010, AH, J211, GL, 3010, CY3, Hf2110 were provided by Henan Academy of Agricultural Sciences, all the names of varieties were registered in China, not hybrids and breeding lines.

Main reagents: atrazine technical material (99.47%) (National University Science and Technology Park, University of Shanghai for Science and Technology, China. Lot#: CTA172); N-N-Dimethylformamide (Tianjin Fuyu Fine Chemical Co., Ltd., China. Lot#: GB/T 17521-1998); dimethyl sulfoxide (Tianjin Fuyu Fine Chemical Co., Ltd., China. Lot#: Q/12HG 3144-2009); Tween 80 (Shanghai McLean Biochemical Technology Co., Ltd., China. Lot#: C15071857); acetone (Tianjin Kaitong Chemical Reagent Co., Ltd., China); malathion (Jiangsu Fengshan Group Co., Ltd., China); NBD-CI (Jiangsu Fengshan Group Co., Ltd., China); ethanol (Tianjin Fuyu Fine Chemical Co., Ltd., China. Lot#: GB/T 678-2002); acetonitrile (Tianjin Fuyu Fine Chemical Co., Ltd., China. Lot#: Q/12F Y 0030-2010); watsons drinking water (Guangzhou Watsons Food and Beverage Co., Ltd., China. Lot#: GB 19298); formic acid (Tianjin Kemi Chemical Reagent Co., Ltd., China. Lot#:); Total RNA Isolation Kit (China Bao Bioengineering Co., Ltd.); prime Script™ RT reagent Kit with g DNA Eraser (China Bao Bioengineering Co., Ltd.); TB Green Premix Ex Taq II (China Bao Bioengineering Co., Ltd.); anhydrous ethanol (Tianjin Fuyu Fine Chemical Co., Ltd., China); RNase-free H_2O (China Sangon Bioengineering Co., Ltd.); 0.5% TBA (Shanghai McLean Biochemical Technology Co., Ltd., China. Lot#: C14071768); chloroform (Tianjin Fuyu Fine Chemical Co., Ltd.); isopropyl alcohol (Tianjin Fuyu Fine Chemical Co., Ltd.).

Test equipment: artificial climate box (China Ningbo Laifu Co., Ltd. Model: POX-450A); pipette (Eppendorf GmbH, Germany. Model: Eppendorf); 1/10000 analytical balance (Shanghai Precision Scientific Instrument Co., Ltd., China. Model: JA3003); UV-visible spectrophotometer (Shanghai Tianmei Scientific Instrument Co., Ltd., China. Model: UV2310 II); high-speed refrigerated centrifuge (China Anhui Jiawen Instrument Equipment Co., Ltd. Model: JW-1042); magnetic constant temperature heating stirrer (China Gongyi Yingyu Instrument Factory. Model: DF-101S); electric heating constant temperature water tank (Shanghai Yiheng Technology Co., Ltd., China. Model: DK-8D); LI-6400 portable photosynthetic measurement system (Ningbo Laifu Co., Ltd., China); ultra-high performance liquid chromatography (USA, Agilent Technologies 1290).

2.1. Determination of resistance level of alfalfa to atrazine

seed culture

The germination treatment was tested according to Wu's method (Su et al., 2016). Uniform their seeds were selected and soaked in warm water at about 30°C for 10 min, then they were rinsed with distilled water and were placed in an incubator at 25°C and were soaked for 12h, then were spread flat on a culture dish lined with wet gauze, and were cultured in an incubator in the dark until the seeds turned white for later use.

soil treatment

Soil samples were collected from the field where commonly plant in Xinxiang, China (Benton Harbor: N 113.9351°, E 35.3829°), air dried at room temperature, thoroughly sieved to remove roots and other plant tissue, and were stored until use.

Screening of 60 alfalfa varieties for resistance to atrazine

The medicated soil was prepared by the soil addition method (Su et al., 2016a). 200 g of air-dried sieved soil was placed in a plastic basin (70 mm high × 83 mm in diameter), and 40 mL of pre-prepared atrazine solution of different concentrations was added to the soil to make the atrazine concentration in the soil 0.2 mg/kg and 10 mg/kg, clear water was set as the treatment control, eight germinated seeds were sown in each pot, the pots were transferred to a greenhouse under controlled conditions (30/25℃

day/night, 70%-75% relative humidity, and light intensity of 150 μmol/($m^2 \cdot s^{-1}$). The soil was completely moistened by bottom infiltration, balanced overnight, and mixed thoroughly to prepare medicated soil of different concentrations. The sensitivity of it was initially determined, and the fresh weight inhibition rate of 60 varieties was evaluated after 6 weeks of treatment.

Determination of resistance levels of 13 alfalfa varieties to atrazine

Further toxicity tests were conducted on 13 varieties that were suspected to be sensitive and resistant to atrazine. The atrazine concentrations were set as follows: sensitive: 0.05, 0.10, 0.20, 0.40, 0.80 mg/kg soil; resistant: 5.0, 10.0, 20.0, 40.0, 80.0 mg/kg soil, clear water was set as the treatment control. After 42 days of cultivation, the shoot was cut and weighed for fresh weight and the fresh weight inhibition rate was calculated. the most sensitive J2 and the most resistant SF to atrazine were screened for the next experiment.

2.2. Study on the non-target resistance mechanism of alfalfa to atrazine

Sample processing

The germination treatment was tested according to Wu's method (Su et al., 2016b). SF and J2 were grown in pure soil: nutrient soil: vermiculite = 10:1:1. After 30 days, SF and J2 with similar growth were transferred to nutrient solution (Ma et al., 2019a). Each aviation cup contains 30 mL of nutrient solution and eight plants. After seven days, the nutrient solution is replaced with 0.03 mg/L atrazine, the leaves and root systems of SF and J2 were sampled before and 0.25 d, 1 d, 3 d, 5 d, 7 d and 10 d after pesticide application and were stored in a -80°C refrigerator to measure the chlorophyll a, chlorophyll b and MDA content.

The final concentrations of atrazine were prepared as 0.1, 0.3, 0.5, 1.0, and 2.0 mg/kg, photosynthetic characteristics and chlorophyll fluorescence parameters of SF and J2 were measured 42 days after application. Each treatment was repeated three times.

Determination of chlorophyll a and chlorophyll b content

The method described by Wang et al (Wang et al., 2017) was modified. 0.05 g of

SF and J2 leaves were weighed and placed in test tubes, 20 mL of acetone and anhydrous ethanol (1:1) mixed extract was added and the leaves were shielded from light for 48 h until they turned white, the test tubes were shaken 1-2 times. 1 mL of supernatant was taken and the OD value was measured at 663 nm and 645 nm on a spectrophotometer. Clear water was used as a control. According to the formula (2-11, 2-12, 2-13, 2-14). Calculate the chlorophyll a and b contents.

Chlorophyll a concentration (Ca) = $12.7A_{663}$ - $2.59A_{645}$ (Formula 2-11);

Chlorophyll b concentration (Cb) = $22.9A_{645}$ - $4.67A_{663}$ (Formula 2-12);

Chlorophyll a content (mg/g FW) = (Ca × total amount of extract) / (fresh weight of sample×1000) (Formula 2-13);

Chlorophyll b content (mg/g FW) = (Cb × total amount of extract) / (fresh weight of sample×1000) (Formula 2-14).

Malondialdehyde (MDA) content determination (TBA method)

The method described by Zhang (Zhang et al., 2021) was modified, 0.2 g of it was weighed, liquid nitrogen was added and ground into powder, 2 mL of pre-cooled 5% TCA grind was added, and then it was put into a centrifuge tube and centrifuged at 4°C, 5,000 rpm for 20 min. The supernatant was aspirated into a new centrifuge tube, an equal amount of 0.5% TBA was added after mixing, boiled in 100°C boiling water for 30 min, cooled to room temperature and centrifuged again at 4°C, 5,000 rpm for 20 min. 0.5% TBA was used as a control for zero adjustment, and the absorbance values of the supernatants in different treatment tubes were measured at 450, 532 and 600 nm in a uv spectrophotometer. MDA content was calculated according to the following formula:

MDA content (μmol/L) = $6.45\times(OD_{532}-OD_{600})-0.56\times OD_{450}$ formula.

Gas exchange measurements

This experiment used Zhu's method (Zhu et al., 2024). The LI-6400 portable photosynthetic measurement system was used to measure the net photosynthesis of alfalfa leaves, under the conditions of the optimal environment (CO_2 concentration: 400 μmol/mol, leaf chamber temperature: 28±0.5°C, light intensity: 150μmol (photon)/($m^2 \cdot s^{-1}$),

Pn, Gs, Tr, and Ci were measured. The measurement time was selected from 9:00 to 11:00 am on a sunny day, and each experimental treatment was repeated four times.

Chlorophyll fluorescence parameters

The leaves to be tested were wrapped in tin foil for dark treatment, after the samples were dark-adapted for one hour, measurements of Fv/Fm, Fv/Fo, Y(II), ETR, qP, and NPQ were taken (Lazár 2015).

2.3. Synergistic effects of enzyme inhibitor malathion and NBD-CI on atrazine

Plant culture

SF and J2 were grown in pure soil: nutrient soil: vermiculite = 10:1:1. After 30 days, the plants were taken out with roots and rinsed with clean water, they with similar growth were transferred to nutrient solution (Ma et al., 2019a). Each aviation cup contains 30 mL of nutrient solution and eight plants. After seven days, SF and J2 were placed in nutrient solution containing atrazine, malathion, NBD-CI, atrazine+malathion and atrazine+NBD-CI, respectively, their fresh weight was measured after 42 days of application, change the solution every two days, set up a water control, and repeat each treatment three times.

Effects of malathion and NBD-CI on the growth of SF and J2

The effects of malathion and NBD-CI on the growth of alfalfa were evaluated. According to the preliminary experimental results, the final concentrations of malathion were set to 6.75, 12.50, 25.00, 50.00, 100.00 and 200.00 mg/kg, and the concentrations of NBD-CI were set to 0.0625, 0.125, 0.25, 0.50 and 1.00 mg/kg respectively, the shoots of SF and giant J2 were cut 21 days after application, and their fresh weights were weighed. The fresh weight inhibition rate was calculated to obtain the highest safe doses of malathion and NBD-CI for alfalfa SF and J2. The highest safe doses of malathion and NBD-CI for two were obtained.

Synergistic effects of malathion and NBD-CI *on atrazine*

To evaluate the synergistic effect of malathion and NBD-CI on atrazine. Preliminary trials confirmed that the highest safe doses of malathion and NBD-CI for SF and J2 were 25 mg/kg and 0.25 mg/kg, respectively. Therefore, the malathion concentration

applied was 25 mg/kg. Atrazine at different concentrations was sprayed 30 minutes after malathion spraying, and NBD-CI at a concentration of 0.25 mg/kg was applied. Atrazine of different concentrations was sprayed 2 days after malathion NBD-CI was sprayed, and the control group was treated with clean water, the shoots were cut 21 days after the drug was applied to determine the fresh weight, and the IC_{50} and RI values were calculated.

2.4. Study on the differences in absorption and metabolism of atrazine by alfalfa

Plant culture

SF and J2 seeds were grown in pure soil: nutrient soil: vermiculite = 10:1:1. After 30 days, they were transferred to nutrient solution (Ma et al., 2019b). Nutrient solution composition is: Ca $(NO_3)_2 \cdot 4H_2O$ 945 mg/L, KNO_3 506 mg/L, NH_4NO_3 80 mg/L, KH_2PO_4 136 mg/L, $MgSO_4$ 241 mg/L, FeNaEDTA 36.7 mg/L, KI 0.83 mg/L, H_3BO_3 6.2 mg/L, $MnSO_4 \cdot H_2O$ 16.9 mg/L, $ZnSO_4 \cdot 7H_2O$ 8.6 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg/L, $CuSO_4 \cdot 5H_2O$ 0.025 mg/L, $CoCl_2 \cdot 6H_2O$ 0.025 mg/L, total 1977.53 mg/L, pH (25℃) 5.8±0.2. In an aviation cup, 30 mL nutrient solution and eight plants were put in each cup. After seven days, it was put into nutrient solution containing 0.03 mg/L atrazine and cultured hydroponically. The atrazine solution was changed every two days, and samples of their shoots and roots were taken at 0.25d, 1d, 3d, 5d, 7d and 10d after application to detect the absorption of atrazine and its metabolites. After ten days of plant application, the plants with consistent growth were transferred to aviation cups filled with nutrient solution with eight plants. On the 0d, 0.25d, 1d, 3d, 5d, and 7d, when the nutrient solution was placed, ten days shoots and root samples of alfalfa were taken to detect the metabolism of atrazine and its metabolites.

Sampling method

Eight plants in each pot were removed from the hydroponic solution, quickly rinsed with clean water, dried with filter paper, and the stems, leaves and roots were separated and cut into small segments of about 1 cm. The entire process was carried out on tin foil cooled by liquid nitrogen. Each repeated sample was mixed and placed in a cryovial and stored at -80°C.

Sample preparation

1.0g SF and J2 were weighed, and the alfalfa shoots or root samples ground by liquid nitrogen were placed in a 50 mL centrifuge tube, 5.0 mL water and 10.0 mL 1% acetic acid acetonitrile were added, vortexed for 1 minute, 1.0 g sodium chloride was added, vortexed for 1 minute, ultrasonicated for 30 min, centrifuged at 4000 r/min for 10 min, 1.5 mL of supernatant was drawn, 50 mg Carb and 50 mg PSA and 100 mg anhydrous magnesium sulfate were added to the centrifuge tube in advance, and centrifuged at 12000 r/min for 5 min, 1mL of supernatant was taken out, passed through a 0.22 um organic filter membrane, and waited for UPLC-MS/MS detection.

Instrument testing conditions

Ultra-high-performance liquid chromatography (Agilent Technologies 1290, USA) conditions: 2 μL of the extract was injected into a column (Agilent C18, 1.8 μ m, 2.1×50 mm) maintained at 25℃ at a flow rate of 0.3 mL/min. The mobile phase was phase A (0.1% formic acid water) and phase B (acetonitrile).

Mass spectrometry (Agilent Technologies 6470, USA) conditions: ion source: ESI+; monitoring mode: multiple reaction detection (MRM). The capillary voltage was 3500 V, the nebulizer gas temperature was 300℃, the nebulizer flow rate was 5 L/min and the sheath gas temperature was 250℃.

Testing of actual samples

Residue detection in shoots and roots of cultured SF and J2 alfalfa based on the method developed above.

2.5. Comparative analysis of transcriptomes of SF and J2 under atrazine stress

Cultivation and treatment of SF and J2

SF and J2 with similar growth potential were transferred to a nutrient solution (Ma et al., 2019c). In an aviation cup, 30 mL nutrient solution and eight alfalfa plants are put in each cup. After seven days, it was put into nutrient solution containing 0.03 mg/L atrazine and cultured hydroponically. The shoots of SF and J2 were sampled 3 days after application, and was stored in a -80℃ refrigerator, the samples were kept for total RNA extraction. Each treatment was repeated three times.

mRNA Sequencing

This experiment used Chen's method (Chen et al., 2021). An appropriate amount of SF and J2 ground by liquid nitrogen was taken and lysed in a 1.5mL centrifuge tube pre-cooled with 600 μL Buffer EL, it was centrifuged (4℃, 13000 g, 5 min). The supernatant was taken and 0.5 times the volume of the filtrate was added with anhydrous ethanol. After shaking, it was centrifuged again (4℃, 13000 g, 15 min). The upper aqueous phase was aspirated and 700 μL Buffer RWA was added. It was centrifuged (4℃, 13000 g, 10 min). The supernatant was discarded, 500ul Buffer RWB was added and it was centrifuged again (4℃, 12000 g, 5 min). The supernatant was discarded, and 50 μL of RNase-free ddH_2O was added for dissolution, mRNA can be separated from total RNA for analysis of transcriptome information.

Library construction and sequencing

Library construction and sequencing were performed according to Hu's method (Hu et al., 2021). High-quality RNA samples were purified and library constructed using the Truseq TM RNA Kit (Illumina, CA, USA). Oligo(dT)-loaded magnetic beads were used to perform A-T base pairing with eukaryotic mRNA with a poly-A tail, and then cleaved into fragments of approximately 300 bp in length using an ion-interrupting fragmentation buffer. Under the action of reverse transcriptase, six-base random primers were added to reversely synthesize the first-strand cDNA using mRNA as a template, and then the second strand was synthesized to form a stable double-stranded structure. PCR was then used for amplification to construct a cDNA library. Finally, it was sequenced on the Illumina Nova Seq6000 platform.

Sequencing data quality control

In order to obtain high-quality sequencing data to ensure the smooth progress of subsequent analysis, the raw sequencing data needs to be filtered. The specific steps and order are as follows:

1. Remove adapter sequences from reads;

2. Trim the bases with a quality value less than 30 at the 3’ end of the sequence;

3. Remove reads containing more than 10% of uncertain base information;

4. Discard sequences whose length was less than 50 bp after adapter removal and quality trimming.

Alignment to the reference genome

The genome alignment was performed according to Zhao's method (Zhao et al., 2023). Sequencing fragments were randomly interrupted by mRNA. In order to determine which genes these fragments were transcribed from, the clean reads after quality control need to be aligned to the reference genome. HISAT2 was used to align the clean reads with the reference genome to obtain the position information on the reference genome or gene, as well as the sequence feature information unique to the sequencing sample. The algorithm of HISAT2 was mainly divided into three parts:

1. Align the entire sequence sequence to a single exon of the genome;

2. Align the sequencing sequence segmentally to the two exons of the genome;

3. Align the sequenced sequences segmentally to more than three (including three) exons of the genome.

expressed genes Expression analysis

Gene expression analysis was performed according to Sui's method (Sui et al., 2022). The abundance of transcripts reflects the expression level of genes. The higher the abundance of transcripts, the higher the expression level of genes. In RNA-Seq analysis, gene expression levels were calculated by counting the number of sequences (clean reads) that map to genomic regions. Use the software RSEM to quantitatively analyze the expression levels of genes and transcripts respectively, so as to subsequently analyze the differential expression of genes/transcripts between different samples, and reveal the regulatory mechanism of genes by combining sequence function information. The results of expression quantification were in units of FPKM, and the normalization process of FPKM maked the total expression in different samples consistent.

In order to screen and identify differentially expressed genes (DEGs), principal component analysis (PCA) was performed on the expression levels of all genes in each sample. Subsequently, DESeq was used for analysis, and the p-value was corrected to obtain the q-value. All differentially expressed genes were divided into up-regulated

and down-regulated DEGs. Through comprehensive analysis of the differential expression levels and functional annotations of DEGs, DEGs induced by atrazine were screened out, mainly including photosynthetic carbon utilization and metabolism, Metabolic pathways such as amino acid synthesis and UDP galactosyltransferase.

2.6. Field application of atrazine in alfalfa plants and soil

Conditions

The experimental field is located in Pingyuan, Xinxiang, China, the following indicators were determined: pH=8.4, organic matter content - 5.5 g/kg, available nitrogen - 29.8 mg/kg, available phosphorus - 6.5 mg/kg and available potassium - 78.3 mg/kg. Xinxiang has a temperate-subtropical climate and a humid monsoon zone. The soil has strong cohesiveness, good water and fertilizer retention properties, and high available nutrients.

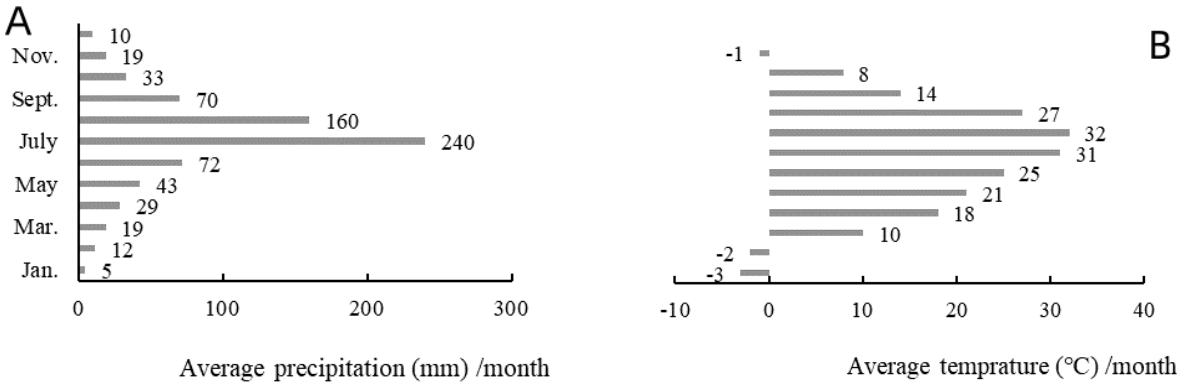

Figure 2.1. Long-term monthly average temperature and precipitation in the study area (Henan Academy of Agricultural Sciences Experimental Field).

The meteorological conditions during the study period were close to the long-term average with a certain trend of warming and drought. Figure 2.1(A) gives average long-term data on monthly temperature and precipitation dynamics during the growing season.

As shown in Figure 2.1(B), alfalfa sowing in China generally starts in the cold dew season of the 24 solar terms, it is mid-to-early October each year. At this time, summer corn in Xinxiang is in the mature harvest period. The temperature in most areas is high and the precipitation is low, it is conducive to improving the wet soil condition and is

also more favorable for the harvest of autumn crops and the preparation of alfalfa for sowing. The sowing date is close to the same period of previous years or 1 to 4 days in advance. The average temperature in mid-to-early October of the survey was 14.4℃, the annual precipitation was 739.4 mm, the relative humidity was 50-65%, the southwest was 3-5, and the frost-free period was about 219 days. The total index of the annual activity temperature sum (>=10℃) in the region is 27℃. Stable heavy rain was recorded from June 15, 2022 to June 25, 2023. The survey showed that the average temperature in October was 14℃, the average precipitation was 33 mm, and the relative humidity was 60-75%. This month is characterized by low humidity. The average temperature in the winter months ranges from -1°C in December to -3°C in January. The last month is considered the coldest, which is favorable for the growth of winter crops. March is the alfalfa tillering period, with an average temperature of 15.2°C. The average precipitation is 19 mm, and the northwest wind is 6-9, they indicate that proper irrigation is needed during this period. The last month of spring (May) has higher temperatures, with an annual average temperature of 26.4°C, precipitation of 1011.7 mm, and relative humidity reaching more than 85%, and southwest wind of level 4-6. This temperature dynamics provides the possibility of accumulation of the sum of active temperatures. Deviations in the dynamic changes of temperature (especially precipitation) lead to yield fluctuations.

In general, the complex hydrothermal conditions and years of research are beneficial to the growth and development of alfalfa and the formation of a high level of productivity.

Trails by randomized block design

The experimental plots were divided according to the relevant requirements of the "Guidelines for Pesticide Residue Tests NY/T 788-2004". The area of each plot was 30m^2. A protection zone and a control group were set up. Before sowing SF and J2, 15 mg/kg of atrazine active ingredient was applied once and the soil was evenly tilled.

Soil and alfalfa plant sampling

Soil samples were taken at 0d, 1d, 3d, 7d, 14d, 21d, 42d, 60d, 90d, 120d, 150d, and

180d after rotary tillage, and crop samples were taken, SF and J2 plant samples were taken at 21d, 120, and 180d after sowing. The samples were stored at -20℃.

Statistical Analysis

Microsoft excel 2019, DPS v7.05 and GraphPad Prism 9.0 were used for calculation, statistical analysis and graphing. All data were the average of three replicates and two independent experiments, expressed as mean ± standard deviation (SD). DPSv7.05 was used to conduct One-Way ANOVA analysis, and Duncan's new multiple range method was used to compare significant indicators of variance analysis.

Conclusions to Chapter 2

This part of the study shows that the whole plant dose-response method was used to determine the resistance level of 60 alfalfa varieties to atrazine, we investigated the sensitivity of SF and J2 varieties to atrazine, and screened out the most resistant and sensitive alfalfa varieties. Using SF and J2 as research objects, the synergistic effects of P450s inhibitor malathion and GSTs inhibitor NBD-CI inhibitor on different concentrations of atrazine were studied through hydroponic application. Apply atrazine for 42 days, chlorophyll content, MDA content, photosynthetic characteristics, and chlorophyll fluorescence parameters were measured at different time points, and the toxic effects of atrazine on two types of alfalfa were studied.

In addition, we established UPLC-MS/MS to detect the differences in residual levels of atrazine in SF and J2 alfalfa at 0.25d, 1d, 3d, 5d, and 10d. Next, we continued to study the comparative analysis of transcriptomes between SF and J2 varieties under atrazine stress.

Finally, field experiments were conducted to verify the effect of atrazine on the growth differences between two varieties.

Chapter 3
DETERMINATION OF RESISTANCE LEVEL OF ALFALFA TO ATRAZINE

3.1. Screening of 60 alfalfa varieties for resistance to atrazine

The resistance level of 60 varieties to atrazine was measured using the whole plant bioassay (poisonous soil method). The results showed that most alfalfa varieties showed varying degrees of resistance to atrazine, which laid the foundation for the economic development of agricultural production and provided a scientific basis for effective and reasonable weed control methods. At the same time, resistant and sensitive varieties were screened out to provide materials for further exploring the resistance mechanism of it to atrazine.

Through the detection and identification of resistant plants, the distribution and level of resistance can be grasped, providing theoretical basis for the scientific breeding and regional distribution of agricultural plants. The commonly used methods for detecting resistance to barnyard grass include whole plant bioassay (soil toxicity assay), physiological and biochemical assays, and molecular level assays (Yuan et al., 2007; Ghanizadeh & Harrington, 2017; Rigon et al., 2020).

The toxic soil method usually has a long time period, but can be tested in large quantities with good repeatability, so it is mostly adopted by scholars at home and abroad (Matzenbacher et al., 2015). Syngenta has established RISQ (Resistance In Season Quick) detection technology after improving the whole plant bioassay method (Kaundun et al., 2011). In this experiment, the indoor toxic soil method was used to determine the inhibitory effects of 0.2 mg/kg and 10 mg/kg atrazine on the growth of 60 alfalfa varieties (Table 3.1). The results showed that the alfalfa varieties of XL, YK, J2, J601 and 801 were relatively sensitive to atrazine, with yellowing, curling, and wilting on the edges of the leaves. The growth inhibition rate of alfalfa at a concentration of 0.2 mg/kg atrazine was more than 70%. At the same time, the varieties FL, HG, 9720, ZM3, WL168H, YS, SF, and J301 were relatively resistant to alfalfa, with no apparent adverse symptoms on the leaves. At a concentration of 10 mg/kg of

atrazine, the growth inhibition rate of it was below 30%.

Table 3.1

Activity determination results of atrazine on different alfalfa varieties (PPPI, HAAS, 2022)

Variety	Dose (mg/L)	Fresh weight inhibition rate (%)	Variety	Dose (mg/L)	Fresh weight inhibition rate (%)	Variety	Dose (mg/L)	Fresh weight inhibition rate (%)
XL	0.20	72.11b	YS	0.20	7.09wx	WL366	0.20	35.55lm
	10.00	100.00a		10.00	49.88w		10.00	55.32t
YK	0.20	70.11c	8421s	0.20	10.37u	WL343	0.20	40.25i
	10.00	100.00a		10.00	51.21v		10.00	60.22kl
J2	0.20	83.24a	SF	0.20	5.23y	GN5	0.20	30.51r
	10.00	100.00a		10.00	61.24jk		10.00	54.11u
J601	0.20	72.24b	J301	0.20	15.17s	AEGJ	0.20	45.24f
	10.00	100.00a		10.00	68.11d		10.00	55.67t
801	0.20	73.09b	JNDDL	0.20	44.32fg	WL440	0.20	30.29r
	10.00	100.00a		10.00	58.91mno		10.00	43.76t
FL	0.20	8.28vw	HX22-64	0.20	32.11opqr	SDL	0.20	55.21e
	10.00	21.28x		10.00	67.34e		10.00	60.38kl
HG	0.20	7.99vw	HX22-65	0.20	36.67kl	YX	0.20	36.79e
	10.00	15.83y		10.00	58.25nop		10.00	64.23h
9720	0.20	5.23xy	HX22-62	0.20	30.02r	4030	0.20	39.25ij
	10.00	24.13w		10.00	57.11qr		10.00	54.23u
HX22-80	0.20	60.24d	HX22-78	0.20	42.10h	J405	0.20	33.56nop
	10.00	68.88cd		10.00	63.29i		10.00	46.87y
HX22-69	0.20	40.32i	HX22-56	0.20	33.21nop	HX2-66	0.20	33.78mno
	10.00	60.21kl		10.00	60.01l		10.00	48.83x
HX22-77	0.20	42.29h	5020	0.20	34.32mn	HX2-58	0.20	35.43lm
	10.00	65.38g		10.00	58.03opq		10.00	59.03mn
LYL	0.20	37.22kl	SD10-2	0.20	33.27nop	SH	0.20	30.01r
	10.00	69.27bc		10.00	60.94kl		10.00	64.45h
TM	0.20	43.20gh	DT	0.20	40.32i	WL363	0.20	30.01r
	10.00	55.21t		10.00	60.99kl		10.00	54.20u
J995	0.20	44.22fg	J801	0.20	39.73ij	J5010	0.20	31.09qr
	10.00	57.56pqr		10.00	69.99b		10.00	61.98j
J201	0.20	35.23lm	YST	0.20	33.32nop	AH	0.20	40.23i
	10.00	55.35t		10.00	57.48pqr		10.00	45.38z
HS	0.20	38.32gr	JG	0.20	31.19qr	J211	0.20	43.98fgh

	10.00	60.92kl		10.00	58.01opq		10.00	67.23e
SD10	0.20	12.03t	WS	0.20	30.91qr	JL	0.20	32.18k
	10.00	29.67v		10.00	60.93kl		10.00	44.38st
ZM3	0.20	8.89uv	LDN	0.20	37.67k	3010	0.20	37.78e
	10.00	34.21u		10.00	54.43u		10.00	46.38y
WL168H	0.20	10.01u	J806	0.20	32.01opq	CY3	0.20	55.39pqr
	10.00	59.22m		10.00	66.02fg		10.00	59.02mn
Hf2110	0.20	Hf2110	LD	0.20	38.29qr	LDN	0.20	30.19kl
	10.00	44.38st		10.00	56.37s		10.00	66.37f

3.2. Determination of resistance levels of 13 alfalfa varieties to atrazine

Atrazine is not easily volatile or photodegradable, and is mainly absorbed by plants and enters the soil (Gao et al., 2014). Due to its high activity and low residue, it often causes phytotoxicity to subsequent crops, affecting crop growth and development, resulting in reduced crop yield and quality, and seriously damaging the economic interests of growers.

The indoor potting method (toxic soil method) was used to determine the inhibitory effect of atrazine on the growth of 13 types of alfalfa (Table 3.2). Among them, under the soil concentration of atrazine 5-160 mg/kg, the growth inhibition rate of the suspected resistant FL, SF, HG, 9720, J301, WL168H, ZM3 and YS increased with the increase of atrazine concentration. Some plants died only after 14 days, and all plants died only after 21 days. The growth of suspected sensitive J601, XL, YK, J2, and 801 increased with atrazine at a soil concentration of 0.025-0.8 mg/kg. As the concentration increases, alfalfa plants appear to be dwarfed or even die within this concentration range.

Research has shown that herbicide residues of herbicides such as Atrazine can have toxic effects on various plants including cucumber (*Cucumis sativus*), peanut (*Glycine max*), soybean (*Arachis hypogaea*), millet (*Setaria italica*), rice (*Oryza satia*) and so on (Chowdhury et al., 2020). Our research also found that atrazine has toxic effects on different alfalfa varieties, but different alfalfa varieties have different resistance to atrazine, this is consistent with previous studies on the different resistance of various soybean varieties to atrazine (Shen et al., 2011). The possible reason is that plants produce a series of reactions in tissue structure, physiology, and biochemistry, among

which various metabolic enzyme activities are most active, and these changes are closely related to plant resistance.

Table 3.2

Activity determination results of atrazine on different alfalfa varieties (PPPI, HAAS, 2022)

Variety	Dose (mg/L)	Fresh weight inhibition rate (%)	Variety	Dose (mg/L)	Fresh weight inhibition rate (%)	Variety	Dose (mg/L)	Fresh weight inhibition rate (%)
	5	34.25a		5	30.00ab		5	43.95a
	10	37.16a		10	43.64c		10	58.09ab
FL	20	61.66b	SF	20	50.07a	WL168H	20	64.70a
	40	68.44bc		40	61.80c		40	72.40bc
	80	68.44bc		80	63.81e		80	76.53cde
	160	90.53b		160	83.07bc		160	84.48bc
	5	33.69a		5	44.33a		5	41.44ab
	10	33.69a		10	47.89ab		10	47.37bc
9720	20	60.85a	ZM3	20	75.18a	HG	20	68.35 a
	40	65.35bc		40	80.32b		40	79.68ab
	80	84.41cd		80	94.50c		80	90.21c
	160	100.00a		160	100.00a		160	100.00a
	5	33.91c		0.025	18.69b		0.025	23.48ab
	10	50.87ac		0.05	33.65ab		0.05	27.68b

J301	20	50.87ac	601	0.1	37.00a	XL	0.1	27.68b
	40	77.97ab		0.2	70.34a		0.2	76.85b
	80	82.74cd		0.4	76.00a		0.4	84.07b
	160	97.52a		0.8	79.91a		0.8	89.55d
	0.025	21.06ab		0.025	32.78a		0.025	32.78c
	0.05	30.07ab		0.05	35.35ab		0.05	35.35c
YK	0.1	30.07ab	J2	0.1	37.20a	801	0.1	37.20b
	0.2	60.15ab		0.2	43.00b		0.2	61.00c
	0.4	60.15ab		0.4	72.84ab		0.4	68.84b
	0.8	88.65ab		0.8	89.91ab		0.8	76.91b
	10	41.27c						
	20	64.70a						
YS	40	69.19bc						
	80	72.48de						
	160	75.78c						

Among them, the toxicity results table shows (Table 3.3) that the IC_{50} of the suspected resistant alfalfa FL, HG, 9720, WL168H, ZM3, YS, SF, and J301 were 6.9832, 14.875, 10.723, 10.6403, 9.8409, 6.459, 9.4103.

respectively. The results showed that SF was highly resistant to atrazine and alfalfa, with an IC_{50} value of 14.8750 mg/kg, the IC_{50} of suspected J601, XL, YK, J2, and 801 was 0.369, 0.4871, 0.460, 0.2428 and 1.288, respectively. The results showed that J2

was more sensitive to atrazine, with an IC_{50} value of 0.2424 mg/kg.

Table 3.3

Toxicity calculation results of atrazine on different alfalfa varieties (PPPI, HAAS, 2022)

Variety	Linear equation	R^2	IC_{50} (mg/kg)
FL	y=4.2517+0.9263x	0.9832	6.9832
9720	y=2.2283+2.699x	0.7874	10.6403
ZM3	y=2.2201+2.8553x	0.8563	9.4103
J301	y=3.6013+1.41085x	0.9679	9.8409
WL168H	y=4.4206+0.7152x	0.9922	6.459
SF	y=4.0574+0.804x	0.9411	14.875
HG	y=2.0161+2.896x	0.8644	10.723
XL	y=5.414+1.3251x	0.9561	0.4871
Yk	y=5.3898+1.1567x	0.9387	0.460
J601	y=5.8418+1.0732x	0.9895	0.369
801	y=4.8629+1.2947x	0.9381	1.288
J2	y=5.3898+0.6333x	0.9958	0.2424
YS	y=4.1574+0.7593x	0.9426	12.873

3.3. Using hydroponic method to determine the sensitivity of SF and J2 to atrazine

Due to the convenient and simple indoor operation of hydroponics, laboratory hydroponics methods are mature. Therefore, the sensitivity of SF and J2 to atrazine was measured again to prepare for future experiments. Due to the widespread use of atrazine, it caused varying degrees of variety and damage to the ecological environment and had varying degrees of impact on the quality and safety of agricultural products such as traditional Chinese medicine, fruits, and vegetables (Cox, 2001; Gammon et al., 2005; Scialli et al., 2014). Our experiment used the hydroponic method to determine the growth inhibition effect of atrazine on the resistant SF and sensitive J2. The results showed that as the concentration of atrazine increased, the fresh weight inhibition rates of SF and J2 gradually increased, and the differences in the fresh weight

inhibition rates were significant.

Table 3.4

Sensitivity test results of SF and J2 to atrazine

(PPPI, HAAS, 2022)

Variety	Dose (mg/L)	Fresh weight inhibition rate (%)	variety	Dose (mg/L)	Fresh weight inhibition rate (%)
SF	1.00	13.18f	J2	0.0625	23.91f
	2.00	26.08e		0.125	38.36e
	4.00	34.38d		0.25	53.45d
	8.00	40.23c		0.50	65.64cd
	16.00	56.96b		1.00	69.09b
	32.00	78.16a		2.00	73.14a

Atrazine has a long half-life and bioaccumulation in soil, so it remains in the soil for a long time. Atrazine remaining in the soil can cause damage to the next sensitive crop (Zhang et al., 2019). Due to the cold climate in Henan Province, China, it is not conducive to the degradation of atrazine, which exacerbates the soil residual pesticide damage to the next crop in the production of this herbicide. The toxicity results of this experiment showed that the IC_{50} of resistant alfalfa SF is 8.28, and the IC_{50} of sensitive alfalfa J2 is 0.29, with a resistance multiple of 28.55 (Table 3.5).

In recent years, the planting industry of alfalfa in China has been continuously expanding. The damage of alfalfa to atrazine residues is a problem that needs to be taken seriously and urgently solved in production, it can affect the yield and quality of alfalfa, hindering the development of the alfalfa industry. Therefore, breeding herbicide tolerant crops can solve the problem of sensitive crops being prone to herbicide residue damage, increase crop safety, improve yield, quality, and economic benefits. The promotion and cultivation of herbicide tolerant crops will become an inevitable path for promoting agricultural development in China.

Table 3.5

Toxicity results of atrazine on alfalfa varieties SF and J2 (PPPI, HAAS, 2022)

Variety	Linear equation	R^2	IC_{50} (mg/kg)
SF	y=3.8932+1.2057x	0.9915	8.28
J2	y=5.4665+0.8705x	0.9988	0.29

Conclusions to Chapter 3

When using the soil toxicity method to determine the resistance level of 60 collected alfalfa varieties to atrazine, the inhibition rate of fresh weight was different for different alfalfa varieties. There were differences in the sensitivity of different alfalfa varieties to atrazine.

When conducting toxicity tests on the 13 preliminarily screened alfalfa varieties against atrazine, the SF with the highest resistance to atrazine was selected, with an IC_{50} of 14.8750 mg/kg, and the J2 with the highest sensitivity had an IC_{50} of 0.2424 mg/kg. SF showed the highest resistance to atrazine, while J2 was the most sensitive to atrazine.

As the concentration of atrazine increased, the fresh weight inhibition rates of both gradually increased, and there were differences between the fresh weight inhibition rates. There was a significant difference in the resistance of SF and J2 to atrazine.

Based on the results of the fourth chapter, 1 Ukrainian Journal was published (Zhu & Rozhkova, 2024).

Chapter 4

STUDY ON THE NON-TARGET RESISTANCE MECHANISM OF ALFALFA TO ATRAZINE

4.1. Synergistic effects of enzyme inhibitor herbicides malathion and NBD-CI on atrazine

Effects of malathion and NBD-CI on the growth of SF and J2

Enhanced metabolic capacity in nontarget resistance was considered to be the main reason for weed resistance to herbicides, which mainly involves a variety of essential enzymes in plants, namely GSTs, P450s, or other mixed-function oxidases and arylacyl amidases wait (Ghanizadeh & Harrington, 2017; Jugulam & Shyam, 2019; Zhan et al., 2024). Among them, many studies on P450s and GSTs are the most critical multifunctional enzymes in the detoxification process (Yuan et al., 2007; Torra et al., 2021; Hwang et al., 2023). Based on the perspective of non-target resistance mechanism, this chapter selects SF and J2 as research objects to determine the synergistic effects of GSTs inhibitors and P450s inhibitors on atrazine and further clarify whether GSTs and P450s are involved in the occurrence of resistance.

Table 4.1

Effects of malathion on the growth of alfalfa varieties SF and J2 (PPPI, HAAS, 2023)

Herbicides	Dose (mg/kg)	Average fresh weight (g)	Herbicides	Dose (mg/kg)	Average fresh weight (g)
Malathion	CK(SF)	0.25±0.03a	Malathion	CK(J2)	0.29±0.09a
	6.75	0.25±0.02a		6.75	0.29±0.11a
	12.50	0.24±0.04ab		12.50	0.27±0.10ab
	25.00	0.23±0.01abc		25.00	0.26±0.02abc
	50.00	0.20±0.04bc		50.00	0.25±0.07bc
	100.00	0.17±0.07c		100.00	0.20±0.07bc
	200.00	0.16±0.07d		200.00	0.13±0.06d

Note: Data expressed as mean ± standard error, lowercase letters indicate the

significance of the difference tested by DPS (Duncan, $p<0.05$). CK: without other treatment.

This experiment evaluated the effects of malathion on SF and J2 (Table 4.1). The experiment found that without the application of malathion, the average fresh weight of SF and J2 was 0.25 g and 0.29 g, respectively. When SF alfalfa was applied with malathion doses of 6.75 mg/kg, 12.5 mg/kg, and 25.00 mg/kg, the average fresh weight was 0.25 g, 0.24 g, and 0.23 g, respectively. There was no significant difference between them and CK, but at 50.00 mg/kg, the average fresh weight was 0.20 g, it was significantly different from CK. When J2 was administered with doses of malathion at 6.75 mg/kg, 12.5 mg/kg and 25.00 mg/kg, the average fresh weight was measured to be 0.29 g, 0.27 g, and 0.26 g, respectively, with no significant difference from CK. However, at 50.00 mg/kg, the average fresh weight was 0.25 g, which was significantly different from CK. Therefore, the experiment proved that the highest safe dose of malathion for SF and J2 was 25.00 mg/kg, and there was no significant difference compared to the control. This is consistent with the results of previous research (Christopher et al., 2001).

Table 4.2

The effect of NBD-CI on the growth of alfalfa varieties SF and J2 (PPPI, HAAS, 2023)

Herbicide	Dose (mg/kg)	Average fresh weight (g)	Herbicides	Dose (mg/kg)	Average fresh weight (g)
NBD-CI	CK(SF)	0.14±0.03a	NBD-CI	CK(J2)	0.14±0.02a
	0.0625	0.13±0.05a		0.0625	0.14±0.12a
	0.125	0.12±0.01ab		0.125	0.13±0.02ab
	0.25	0.11±0.07abc		0.25	0.11±0.04abc
	0.50	0.09±0.05c		0.50	0.10±0.09cd
	1.00	0.06±0.04d		1.00	0.08±0.04d

Note: Data expressed as mean ± standard error, lowercase letters indicate the significance of the difference tested by DPS (Duncan, $p<0.05$). CK: without other

treatment.

To evaluate the effects of NBD-CI on SF and J2 (Table 4.2). The experiment found that when NBD-CI was not applied, the average fresh weight of SF and J2 was 0.14 g and 0.14 g, respectively. When SF alfalfa was treated with malathion at doses of 0.0625 mg/kg, 0.125 mg/kg, and 0.25 mg/kg, the average fresh weight was 0.13 g, 0.12 g, and 0.11 g, respectively. There was no significant difference between them and CK, but at 0.50 mg/kg, the average fresh weight was 0.09 g, it was significantly different from CK. When J2 was treated with malathion at doses of 0.0625 mg/kg, 0.125 mg/kg, and 0.25 mg/kg, the average fresh weight was measured to be 0.14 g, 0.13 g, and 0.11 g, respectively, there was no significant difference from CK. However, at 0.50 mg/kg, the average fresh weight was 0.10 g, which was significantly different from CK. Therefore, experiments have shown that the highest safe dose of malathion for SF and J2 was 0.25 mg/kg, and there was no significant difference compared to the CK. Therefore, the experiment proved that the highest safe dose of NBD-CI for SF and J2 was 0.25 mg/kg, and there was no significant difference compared to the control. This is consistent with the results of previous research (Ndo et al., 2019).

The synergistic effect of malathion on atrazine

Plant cytochrome P450s enzymes play a crucial role in detoxification pathways and can achieve detoxification of herbicides through metabolism (Morant et al., 2003). Metabolizing and detoxifying herbicides through P450s was an important herbicide resistance mechanism evolved by some herbicide resistant weed populations. The current understanding of P450s mediated herbicide metabolism in plants suggests that there may be multiple P450s subtypes with different herbicide (substrate) specificity, therefore the ability of herbicide metabolism was also limited (Schuler et al., 2003).

The experiment followed the method of Délye (Délye et al., 2002a) to evaluate the synergistic effect of malathion on atrazine. Pre-test verification showed that the highest safe dose of malathion for alfalfa was 25 mg/kg. Therefore, the concentration of malathion applied in this experiment was 25 mg/kg. When using atrazine alone, the IC_{50} of SF was 8.28, and the IC_{50} of J2 was 0.29. Compared with using atrazine alone,

the IC_{50} of atrazine combined with malathion decreased significantly. The IC_{50} of SF alfalfa was 2.74, the IC_{50} of J2 was 0.13, and the IC_{50} ratio of SF and J2 was 3.02 and 2.21, respectively (Table 4.3). Therefore, it was speculated that the resistance of SF and J2 to atrazine may be related to P450s.

Similarly, Burnet (Burnet et al., 2011) reported that the main mechanism of ryegrass resistance to simazine was due to the increased activity of P450s, which leads to an accelerated metabolic rate of resistant ryegrass. Svyantek (Svyantek et al., 2016) found that target gene mutations were one of the reasons for the development of drug resistance in poa pratensis. At the same time, in resistant poa pratensis that did not undergo mutations, enhanced P450s activity can also cause poa pratensis to develop resistance to simazine, atrazine, zopiclone, dichlorvos, and azoxystrobin. Our experimental results were consistent with these results.

Table 4.3

Differences in the resistance level of different alfalfa varieties to atrazine after application of malathion (PPPI, HAAS, 2023)

Variety	IC_{50} (mg/kg)	IC_{50} (mg/kg)	IC_{50} value ratio
	Atrazine (A)	Atrazine＋malathion (B)	A/B
SF	8.28±0.19a	2.74±0.27b	3.02
J2	0.29±0.05a	0.13±0.01b	2.21

Note: A: Spraying atrazine, B: Spraying atrazine + malathion, data expressed as mean ± standard error, lowercase letters indicate the significance of the difference tested by DPS (Ducan, $p<0.05$).

The synergistic effect of NBD-CI on atrazine

GSTs was a multifunctional enzyme family that was commonly found in animals and plants. GSTs participated in detoxification metabolism by binding glutathione with herbicides to form hydrophilic complexes and transporting them outside the cell membrane. GSTs had high catalytic activity and protects crops from herbicide damage by catalyzing the binding of triazene herbicides to glutathione (Wang et al., 2017).

Table 4.4

Differences in the resistance level of different alfalfa varieties to atrazine after application of NBD-CI (PPPI, HAAS, 2023)

Variety	IC_{50} (mg/kg)	IC_{50} (mg/kg)	IC_{50} value ratio
	Atrazine (A)	Atrazine＋NBD-CI (B)	A/B
SF	8.28±0.19a	2.74±0.27b	1.08
J2	0.29±0.05a	0.13±0.01b	1.21

Note: A: Spraying atrazine, B: Spraying atrazine + NBD-CI, data expressed as mean ± standard error, lowercase letters indicate the significance of the difference tested by DPS (Ducan, $p<0.05$).

The experiment followed the method of Délye (Délye et al., 2002b) to evaluate the synergistic effect of NBD-CI on atrazine. Pre-test verification showed that the highest safe dose of NBD-CI for alfalfa was 0.25 mg/kg. Therefore, the concentration of NBD-CI applied in this experiment was 0.25 mg/kg. When using herbicide alone, the IC_{50} of SF was 8.28, and the IC_{50} of J2 was 0.29. Compared with using herbicide alone, the IC_{50} of herbicide combined with malathion decreased significantly. The IC_{50} of SF alfalfa was 7.67, the IC_{50} of J2 was 0.24, and the IC_{50} ratio of SF and J2 was 1.08 and 1.21, respectively (Table 4.4). Therefore, it was speculated that the resistance of SF and J2 to herbicide may have nothing to do with GSTs. Previous studies have found that the combination of GSTs transferase inhibitor NBD-CI and atrazine does not significantly reduce the fresh weight of weeds (Shergill et al., 2018), Prado (2000) found no similarities or differences in the absorption and metabolism of atrazine between Setaria viridis and Setaria faberii, two biotypes, through their study of metabolic pathways against atrazine. These results were consistent with the findings of this experiment(Prado et al., 2000), these results were consistent with the findings of this experiment.

4.2. The effect of atrazine on the growth of SF and J2

Studying the impact of soil residued on subsequent crops is crucial. For example, bensulfuron-methyl had toxic effects on the growth and development of cucumbers and chlorophyll fluorescence parameters (Sun et al., 2019), while bensulfuron methyl also

had serious inhibitory effects on soybeans and peanuts, ultimately resulting in a decrease in plant development levels (Su et al., 2018). Atrazine treatment inhibited the growth of SF and J2. With the increase of atrazine residue, PH and RL of J2 significantly decreased. On the 30th day after treatment, under low concentration (0.13 mg/kg), PH and RL of J2 significantly differed from the control. PH and RL decreased by 15.32% and 29.53%, respectively. Under the high concentration (2.00 mg/kg) treatment, alfalfa's plant height and root length significantly differed from the control. PH and RL decreased by 81.83% and 81.73%, respectively, reaching highly significant levels. For SF, when the atrazine concentration was 0.5 mg/kg, PH and RL significantly differed from the control. PH and RL decreased by 35.65% and 20.29%, respectively. And there was a significant difference from J2 (Figure 4.1).

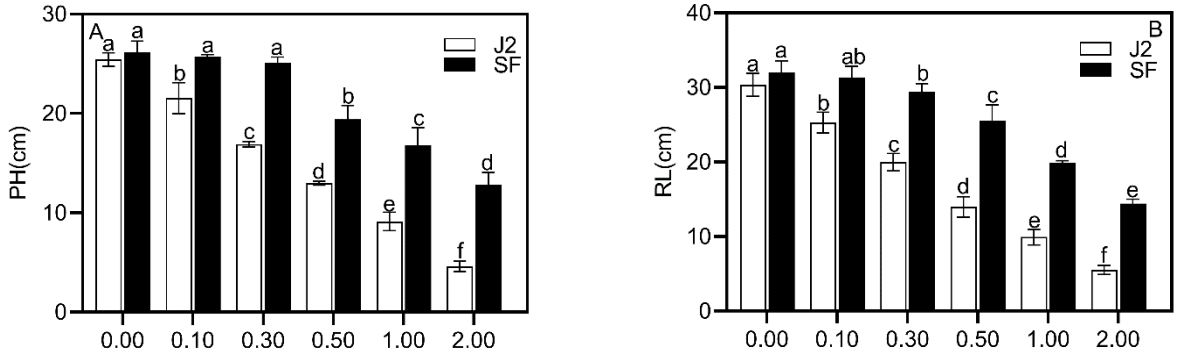

Figure 4.1. Effects of different concentrations on PH (A) and RL (B) of SF and J2 (PPPI, HAAS, 2023)

Atrazine has biological toxicity and acts on the photosynthesis of plants. Therefore, plants in the environment will be directly or indirectly threatened by atrazine, which can inhibit seed germination and plant growth, and in severe cases, lead to fatal crop failure (Feng et al., 2023). Zhang studied the stress damage of atrazine on alfalfa using hydroponic method, after the third true leaf of alfalfa was grown and placed in atrazine nutrient solution for 6 days, the growth and development of alfalfa roots were severely inhibited (Lu et al., 2022).

Our experiment found that the dry weight of sensitive giant energy 2 alfalfa decreased with the increase of atrazine residue (Figure 4.2). When the residual

concentration of atrazine was 0.13-2.0 mg/kg, the soil concentration significantly reduced SDW and RDW of J2. Compared with the control, SDW was reduced considerably by 23.08%-92.31%, and RDW was significantly reduced by 3.13-77.08%. For SF, abnormal symptoms appeared on the plant when the atrazine concentration was 0.50 mg/kg. Compared with the control, SDW and RDW decreased by 35.65% and 63.29%, respectively, and were significantly different from the J2.

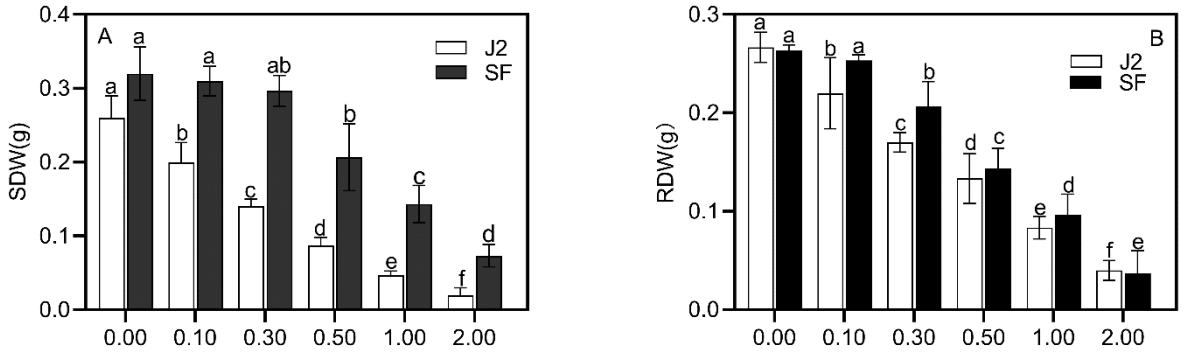

Figure 4.2. Effects of different concentrations on SDW and RDW of SF and J2 (PPPI, HAAS, 2023)

4.3. Effect of atrazine on photosynthetic characteristics and chlorophyll fluorescence parameters of alfalfa

Gas-exchange measurements

The growth and development of plants are closely related to photosynthesis. During drug stress, the influencing factors of plant photosynthesis include stomatal and non-stomatal factors, but during brief drug stress, the main factor affecting plant photosynthesis is stomatal factors (Huang et al., 2012; Soares et al., 2022). This experiment used the LI-6400 portable photosynthetic measurement system, under the conditions of ambient CO_2 concentration of 400 μmol/mol, leaf chamber temperature of 28±0.5°C, and light intensity of 150 μmol (photon)/($m^2 \cdot s^{-1}$), the results showed that as the soil as the atrazine content increases, the Pn, Gs, and Tr of J2 gradually decreased. In contrast, Ci gradually increased (Figure 4.3). At the lowest concentration (0.13 mg/kg), Pn,

Gs and Tr were significantly reduced by 13.95%, 14.01%, and 27.45%, respectively. On the contrary, Ci increased significantly by 21.24%. At a high level (2.00 mg/kg), Pn, Gs, Tr, and Ci changed by 83.42%, 84.15%, 91.57% and 46.69%, respectively. For resistant alfalfa, before the atrazine concentration is 0.5 mg/kg, there was no significant difference between Pn, Gs, Tr, Ci and CK. At 0.5 mg/kg, Pn, Gs, and Tr were significantly reduced by 34.03, 34.00%, 17.90% and 27.59%, Ci increased by 16.38%, and was significantly different from J2.

The results of this experiment indicated that SF and J2, when subjected to continuous atrazine stress, reduce Tr and close stomata in order to maintain water in the plant body. CO_2 cannot enter the leaves, resulting in a decrease in Pn, Gs, and Tr, while Ci gradually increased. This result was consistent with research on soybean (Guo et al., 2015), wheat(Ma et al., 2018), and camphor trees(Luo et al., 2019). During the stress period of atrazine, the Gs and Tr of SF and J2 decreased significantly, while Ci gradually increased, indicating that stomatal resistance caused a decrease in Pn and that they adapted to the environment by increasing Ci under atrazine stress.

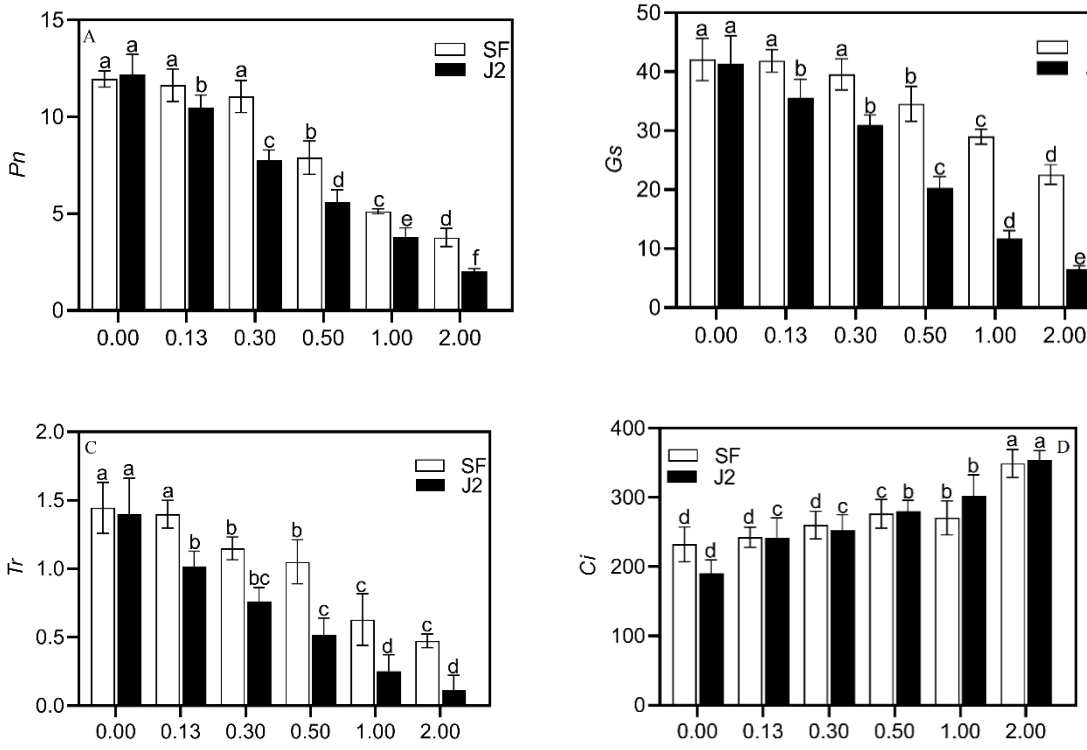

Figure 4.3. (A) Pn, (B) Gs, (C) Tr, (D) Ci (PPPI, HAAS, 2023). Duncan was used to analyze the mean values of each parameter, and the significant difference between treatments marked with different lowercase letters was at $P < 0.05$.

Chlorophyll fluorescence parameters chlorophyll fluorescence parameters

Leaves are the main organs for photosynthesis and transpiration in plants, chloroplasts are the main site for photosynthesis, and stomata are the main channels for transpiration in plants (Gojmerac et al., 2006). The degree of closure directly affects photosynthesis. Photosynthesis is the foundation of plant growth and the most important factor in plant productivity. Plant photosynthesis is highly sensitive to environmental factors and is closely related to species, sources, and varieties. Photosynthesis is the foundation of plant growth and yield formation, with over 90% of crop yield coming from organic matter produced by photosynthesis (Iriel et al., 2013).

Chlorophyll fluorescence parameter is an important indicator for evaluating plant photosynthesis, which can quickly and accurately reflect the absorption, transmission, and conversion of light energy by plants under herbicide stress. Chlorophyll fluorescence was as one of the natural probes in plants, was closely related to plant photosynthesis and can quickly and sensitively characterize the photosynthetic function of plants (Juneau et al., 2007; Hassannejad et al., 2020). There have been many studies on the chlorophyll fluorescence characteristics and changes in the function and structure of photosynthetic organs in plants under various stress conditions. Chris et al. found that the degree of decrease in photosystem II (PSII) activity is related to wheat (*Triticumes tivum*) varieties, with stronger resistant varieties showing a smaller decrease. The research results of Yu et al. showed that under herbicide stress, the primary light energy conversion efficiency and potential activity of PSII in winter wheat were inhibited (Chris et al., 2008; Yu et al., 2023).

This experimental study found that with the increase of atrazine residue, Fv/Fm, Fv/Fo, Y(II), ETR and qP of J2 decreased, while NPQ showed the opposite trend. When the concentration was higher than 0.13 mg/kg, the Fv/Fm, Fv/Fo, Y(II), ETR, and qP

of alfalfa significantly differed from the control. At 0.25 mg/kg concentration, Fv/Fm, Fv /Fo, Y(II), ETR, and qP decreased by 4.71%, 30.81%, 30.86%, 24.92%, and 20.60%, respectively. At the same time, NPQ was significantly different from the control at this concentration and increased by 16.33% at the high concentration of 2.00 mg/kg concentration, Fv/Fm, Fv/Fo, Y(II), ETR and qP decreased by 11.76%, 76.90%, 80.00%, 63.12% and 46.44%, respectively, while NPQ was the same as the control at this concentration. Significantly different, increased by 37.64%. For resistant SF, Fv/Fm, Fv/Fo, Y(II), ETR and qP all decreased at an atrazine concentration of 0.5 mg/kg, while the opposite was true for NPQ at this concentration. When the atrazine concentration was 0.50 mg/kg, the Fv/Fm, Fv/Fo, Y(II), ETR and qP of SF decreased by 10.11%, 8.00%, 24.56%, 26.62%, and 11.11%, respectively, while NPQ increased by 19.72%, which was significantly different from J2 at this concentration (Figure 4.4).

Atrazine is a photosynthetic inhibitory herbicide, whose main target is the electron transporter β protein between PS II and PS I before reduction by plastoquinone. After binding with atrazine, the amino acid structure of the protein is altered, inhibiting electron transfer from plastoquinone QA to QB, thereby affecting photosynthetic electron transfer and ultimately leading to the accumulation of oxygen free radicals and PS II reaction in the plant (Morant et al., 2004). Due to its high selectivity and absorbency, it is one of the preferred herbicides for weed control in corn fields.

The main ways for chlorophyll to absorb light energy and dissipate light energy are through chlorophyll fluorescence, electron transfer, and heat dissipation pathways. Plants subjected to adversity stress can experience temporary photoinhibition or direct damage to their photosynthetic organs, leading to a decrease in chlorophyll fluorescence parameters (Shaw et al., 2008). The results of this experiment indicate that under the corresponding dose of herbicide treatment, the electron transfer chain was blocked during the normal growth process of the corresponding alfalfa, and photosynthesis was affected. When alfalfa plants are subjected to residual herbicide stress in the soil, the decrease in Fv/Fm was directly related to photosynthetic efficiency, carbon metabolism, productivity, and crop growth (Ralph et al., 2005).

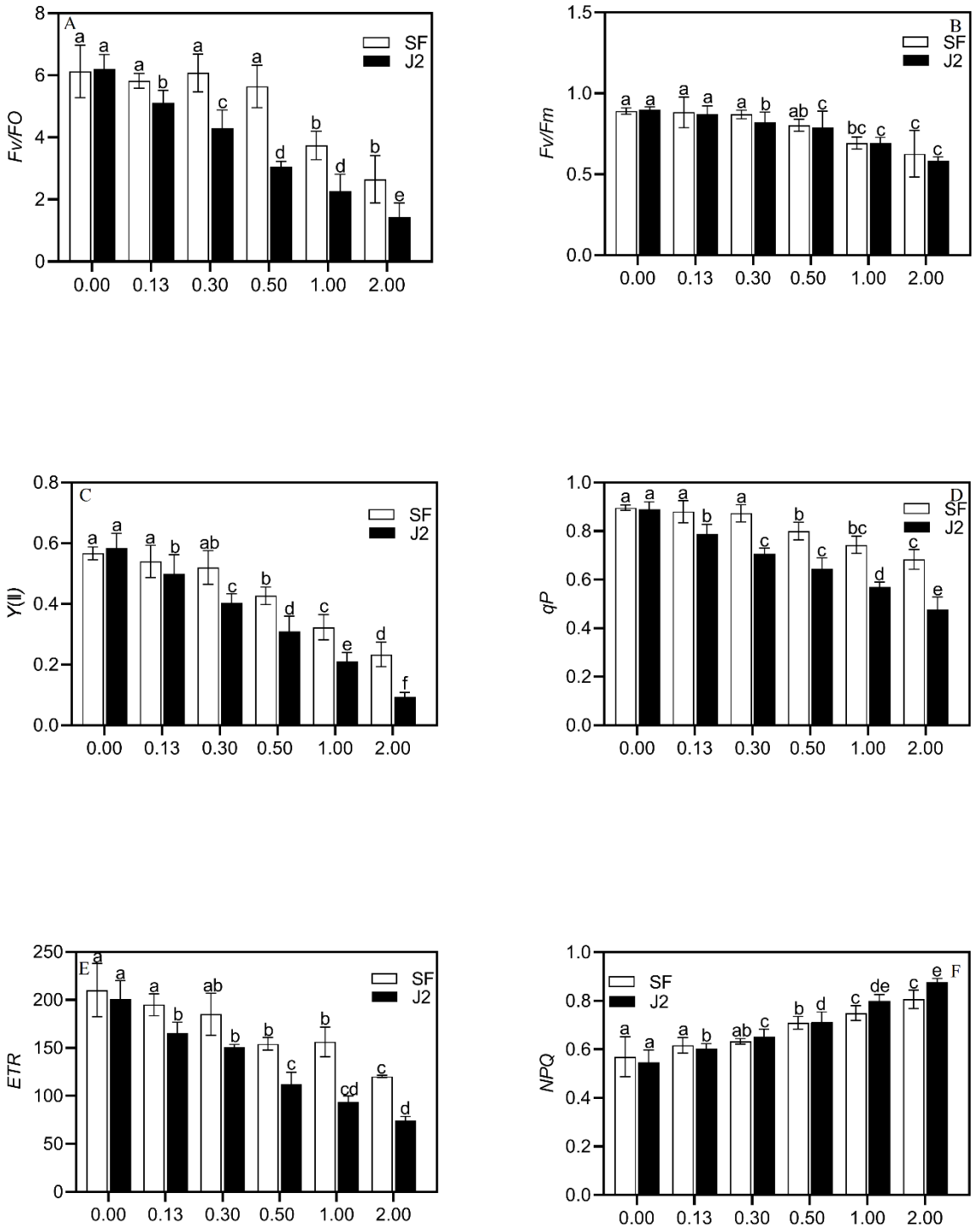

Figure 4.4. Effect of atrazine residue on chlorophyll fluorescence parameters of lucern. (A) Fv/Fo, (B) Fv/Fm, (C) Y(II), (D) qP, (E) ETR, (F) NPQ (PPPI, HAAS, 2023). Duncan was used to analyze the mean values of each parameter, and the significant difference between treatments marked with different lowercase letters was at $P < 0.05$.

4.4. The effect of atrazine on chlorophyll content in alfalfa SF and J2

Photosynthesis refers to the process in which green plants convert light energy into chemical energy using carbon dioxide and water through chloroplasts, store it in organic matter, and release oxygen. The first step of photosynthesis is the absorption of light energy by chlorophyll and the ionization of chlorophyll. The generated chemical energy is temporarily stored in adenosine triphosphate (ATP), converting carbon dioxide and water into carbohydrates and oxygen (Juneau et al., 2007; Dayan et al., 2012). This experiment found that before the application of atrazine, there was no significant difference in the chlorophyll content of SF and J2. After atrazine was used, the contents of SF and J2 gradually decreased with the increase of application days and dropped on the 10th day to the lowest. Compared with the control, the chlorophyll a content of SF and J2 was 0.83 times and 0.22 times that of the power, respectively. Compared with the control, the chlorophyll b content of SF and J2 was 0.72 times and 0.22 times that of the control. The content decreased the fastest within three days. On the third day, the J2 of chlorophyll a content was 0.42 times that of SF and J2 of the chlorophyll b content was 0.31 times that of SF. There was a significant difference between the two.

Chlorophyll is an important photosynthetic pigment in plants, capable of absorbing and transmitting light quanta. In the study of the effects of herbicides on crop photosynthesis, it was found that most herbicides have a certain impact on crop photosynthesis after use, and herbicide treatment of soybeans and rice resulted in a decrease in chlorophyll content (Li & Wu, 2007). Our experiment found that with the prolongation of atrazine stress, SF and J2 were both damaged to varying degrees, confirming that the integrity of thylakoid membranes was compromised, the activity of key enzymes in chlorophyll synthesis pathways decreased, and the activity of enzymes in degradation pathways increased, which were the main reasons for the decrease in chlorophyll concentration (Wang et al., 2015). Chlorophyll is the material basis for photosynthesis in higher plants, and many studies have shown that atrazine can reduce plant chlorophyll content and alter its components (Li et al., 2016).

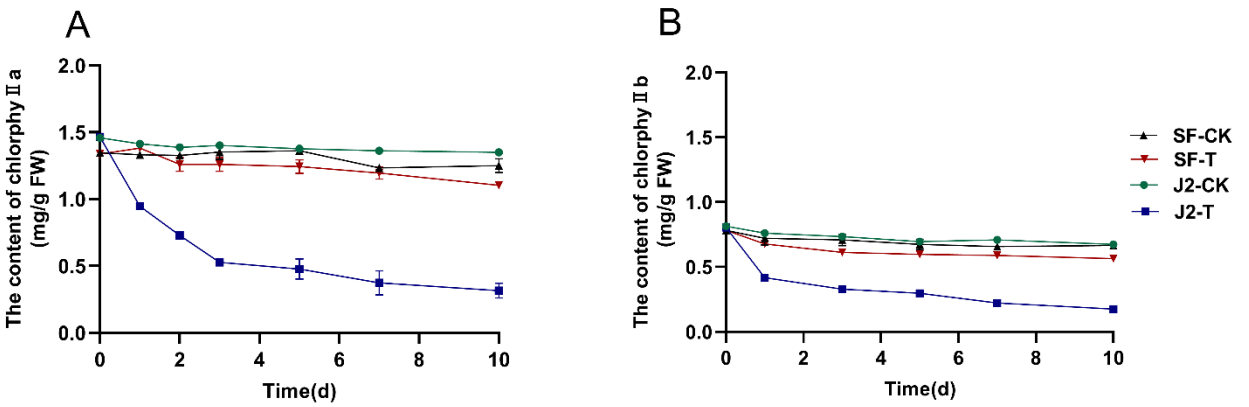

Figure 4.5. Effects of atrazine on chlorophyll a (A) and chlorophyll b (B) content in SF and J2 alfalfa (PPPI, HAAS, 2023)

4.5. Effect of atrazine on MDA content of SF and J2 alfalfa

MDA is the final product of membrane peroxidation, which has a toxic effect on plant cells. Its content reflects the degree of damage to the plant cell membrane, that is, the higher the MDA content, the more reactive oxygen species it produces, the greater the membrane peroxidation reaction, and the greater the damage to the cell membrane (Lukatkin et al., 2013; Alves et al., 2018; Karpenko et al., 2019). The antioxidant enzymes in plants can eliminate the damage of free radicals and other substances to plant cells, maintaining normal growth of plants under adverse conditions. Our experiment found that there was no difference in MDA content between SF and J2 before atrazine application. After applying atrazine, as the number of days increased, the MDA content in the above-ground parts of J2 increased faster within three days. On the third day, the MDA content of J2 was 4.93 times that of the control and 2.01 times that of SF. The growth rate was slightly slower after three days, before three days, the growth rate of MDA content of SF had been increasing slowly. Under the same treatment, there was a significant difference in the MDA content of atrazine between SF and J2.

Under the stress of atrazine, the MDA content in J2 significantly decreased in this experiment, this was consistent with the results of studies on grafted cucumber and rootstock seedlings under self-toxicity (Yu et al., 2018). This was due to the fact that GR can participate in the ascorbic acid glutathione cycle pathway. Enzymes adapted to

stress and the ASA-GSH system in the system work together to remove H_2O_2, thereby enhancing their antioxidant capacity and enabling membrane lipids to resist free radical oxidation, thus protecting the biofilm (Peng et al., 2012).

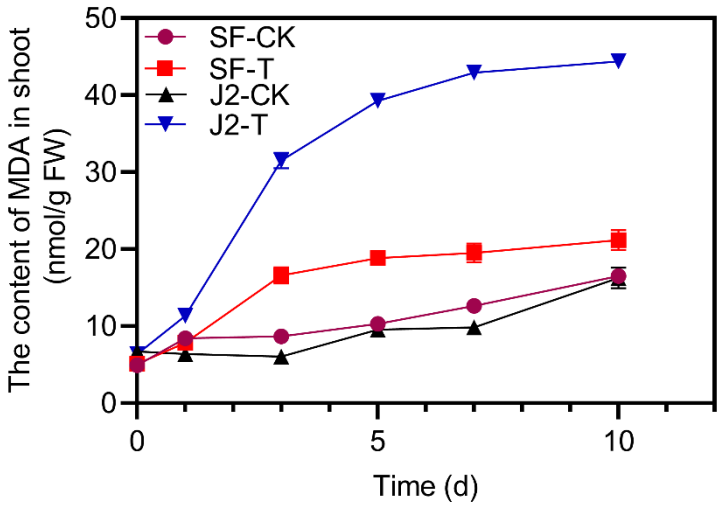

Figure 4.6. Effects of atrazine on MDA content in SF and J2 alfalfa (PPPI, HAAS, 2023)

Conclusions to Chapter 4

Malathion and NBD-CI had inhibitory effects on the fresh weight of SF and J2 alfalfa. The highest safe doses of malathion and NBD-CI for SF and J2 were 25.00 mg/kg and 0.25 mg/kg, respectively, with significant differences between the two.

Malathion had a synergistic effect on atrazine, while NBD-CI had not a synergistic effect. Compared with the use of atrazine alone, the IC_{50} of SF and J2 decreased significantly after treatment with malathion in combination, and there was a significant difference. It was speculated that the resistance of SF and J2 to atrazine may be related to P450s. However, there was no significant difference after combined treatment with NBD-CI, suggesting that the resistance of SF and J2 to atrazine may not be related to GSTs.

SF and J2 were suffered varying degrees of damage under atrazine stress, but the differences between the two were significant. With the increase of atrazine concentration, PH, RL, SDW, and RDW of SF and J2 were all damaged. Moderate

levels of atrazine had no significant effect on SF, but had a significant effect on J2, indicating differences in resistance between SF and J2 to atrazine.

Excessive residue of atrazine inhibited the photosynthesis of SF and J2 to varying degrees. With the increase of atrazine concentration, Pn, Gs, and Tr of SF and J2 alfalfa decreased, while Ci increased, indicating that alfalfa reduced the damage of atrazine to their leaf photosynthetic apparatus by enhancing respiration. The Fv/Fm, Fv/Fo, Y (II), ETR, and qP of SF and J2 decreased, while NPQ increased. This indicated that atrazine stress caused damage to the PSII reaction centers of SF and J2, thereby inhibiting plant photosynthesis.

The residue of atrazine reduced the content of chlorophyll a and chlorophyll b in SF and J2. As the application time of atrazine increased, the content of chlorophyll a and b in J2 decreased significantly compared to CK, indicating that J2 was more sensitive to atrazine. There was no significant difference between SF and the control group. Indicating that SF was more resistant to atrazine.

The residue of atrazine increased the MDA content of SF and J2, causing varying degrees of damage to them. As the application time of atrazine increased, the MDA content of J2 and SF decreased compared to CK, but the difference in content between the two was significant. J2 was more sensitive to atrazine.

Based on the results of the fourth chapter, 1 Ukrainian Journal and 1 Scopus were published (Zhu & Rozhkova, 2023; Zhu & Rozhkova, 2024).

Chapter 5

STUDY ON THE DIFFERENCES IN ABSORPTION AND METABOLISM OF BY ALFALFA

5.1. The chromatographic and mass spectrometry conditions were optimized

Reducing the absorption of herbicides by weeds and enhancing the metabolism of herbicides by weeds are the main non-target mechanisms for weeds to become resistant to herbicides (Suckling & Sforza, 2014; Jugulam & Shyam, 2019). It has been previously confirmed that SF had a high resistance to atrazine. Medicinal properties (IC_{50} was 8.28), J2 was sensitive to atrazine (IC_{50} was 0.29). Therefore, this chapter used UPLC-MS/MS method to clarify further the effects of SF and J2 alfalfa on atrazine absorption and metabolism.

MRM multi reaction monitoring is a highly specific and sensitive mass spectrometry technique that can selectively quantify compounds in complex mixtures. This technology uses triple quadrupole ms, which first targets the ion corresponding to the compound of interest, and then the target ion is fragmented to produce a series of sub ions. One (or more) of these fragment sub ions can be selected for quantitative purposes (Zhong et al., 2022). In ESI positive ion mode, atrazine can obtain stable and sensitive parent ion. Secondary mass spectrum analysis was performed for atrazine, and mass spectrum conditions such as daughter ions, cone-hole voltage and collision energy were optimized. Therefore, the mass spectrum parameters of optimal multiple reaction monitoring mode (MRM) for atrazine were finally obtained (Table 5.1).

Table 5.1

UPLC-MS/MS parameters for atrazine and its compounds (PPPI, HAAS, 2023)

Herbicide	Retention time (min)	Quantitative and qualitative ions (m/z)	Frangmentor (V)	Collision energy (eV)
Atrazine	1.339	216.0>174 216.0>68.2	30 25	17 25

The optimization of mobile phase is helpful to improve the separation efficiency

and sensitivity of the target substance (Wang et al., 2011). The mobile phase consists of A and B. In this experiment, A: acetonitrile and B: water, A: acetonitrile and B: 0.01% ammonia water, A: acetonitrile and B: 0.1% formic acid water was used as mobile phases to investigate the effects on the peak type and response intensity of atrazine. The results showed that when A: acetonitrile and B: water was used as mobile phase, the response intensity of atrazine was low and the chromatographic peak was trailing. When A: acetonitrile and B: 0.01% ammonia was used as mobile phase, the retention time was advanced, the peak shape was obviously improved, and the response value was increased by 10 times. Therefore, this study finally determined the T3 chromatographic column separation, A: acetonitrile and B: 0.1% formic acid water as the mobile phase, mobile phase gradient elution procedure (Table 5.2). Under these conditions, the relative retention times of atrazine on the liquid chromatography-mass spectrometry were approximately 1.366 min.

Table 5.2

Gradient elution procedure (PPPI, HAAS, 2023)

Time	Flow (mL/min)	A/%	B/%
0.00	0.4	100.00	0.00
2.00	0.4	62.00	38.00
2.10	0.4	50.00	50.00
3.00	0.4	20.00	80.00
5.00	0.4	5.00	95.00
6.00	0.4	5.00	95.00

5.2 Optimization of preprocessing methods

Pesticide residue detection can be generally divided into three parts: extraction of the pesticide to be tested, purification and analysis of the pesticide to be tested. Because there are many impurities in the samples collected in the field test, the pesticide to be tested in the laboratory samples should first be extracted and purified to reduce the content of other impurities except the target compound in the samples, so as to meet the detection requirements (Cheng et al., 2023). To reduce the impact of matrix on the

test substance, the effects of three purification agents, C_{18}, PSA, and C_{18}+PSA, on the recovery rate were investigated. when C_{18}+PSA was used as the purification agent, the response intensity of the two test substances was the highest. At the same time, the amounts of C_{18} and PSA were compared, when the amounts of C_{18} and PSA reached 100 mg, the response value of the tested substance reached the highest. Afterwards, the response intensity no longer increased with the increase of adsorbent amount. Therefore, 50 mg C_{18}+50 mg PSA were used as the pre-treatment purification agent. It was the same pre-treatment method as Jia (Jia et al., 2014) in determining the presence of herbicide and nicotinuron in corn. This method was simple to operate, saves organic solvents, accurate, and meets the requirements of pesticide residue analysis in all indicators.

5.3 Linear equations and correlation coefficients

Extract and determine a series of standard solutions of different concentrations under optimal experimental conditions. The regression equation of the standard curve of atrazine obtained in this experiment is y=1541340.7+19861.1, R=0.9999, indicating a good linear relationship of atrazine in the concentration range of 0.05-1.0 mg/kg (Table 5.3). Yang (Yang et al., 2023) used UPLC-MS/MS to detect the residual levels of atrazine and its metabolites in the target substance and found that the standard curve in corn was y=93627x+95.2286, R=0.9992, the standard curve in straw was y=911125x+101.143, R=0.9997. The results of this experiment were consistent with his experimental results, and the experimental methods met the conditions for residual detection.

Table 5.3

Linear regression equation and correlation coefficient of atrazine (PPPI, HAAS, 2023)

Herbicides	Linear equation	R^2
Atrazine	Y=1541340.7+19861.1	0.9999

5.4 Accuracy and precision of the method

With the continuous confirmation of research results on the harm of herbicides to

animals and plants and the threat to human health, people have become increasingly concerned about the residues of herbicides in food and the environment. The research on accurate and reliable residual detection technology of atrazine is an important basis for evaluating the impact of atrazine on food quality, safety, and human health. Therefore, in recent years, researchers from various countries have invested a lot of effort in studying residual detection methods of atrazine in various samples (Ni et al., 2019a).

Regardless of the method used to determine pesticide residues or conduct other experiments, the accuracy and precision of the experimental method should be demonstrated first. Only on the basis of good accuracy and precision can the data or results obtained from experimental methods explain the problem (Ni et al., 2019b). Therefore, add three concentration levels of atrazine mixed Standard solution to the shoot and root blank sample of alfalfa plants to carry out the addition and recovery experiment, and set up five parallel groups for each standard concentration. After vortex mixing, a pre-treatment method was used for UPLC-MS/MS detection to calculate the average recovery rate and relative standard deviations (RSDs).

When the concentration of alfalfa added to the shoot part is within the range of 0.1-5.0 mg/kg, the recovery rate was between 102.6% and 107.7%, and the relative standard deviation was relatively between 2.8% and 6.9%, which can well meet the requirements of residue detection. This indicated that the accuracy and precision of the experimental method were relatively good, both meeting the requirements of residual analysis (Table 5.4). This result was consistent with previous research findings and meets the requirements for residual conditions (Liu et al., 2019).

Table 5.4

The average recovery rate and relative standard deviation of the aboveground methods (PPPI, HAAS, 2023)

Herbicide	Add level (mg/kg)	Recovery (%)	RSD (%)

Atrazine	0.1	107.5	2.8
	0.5	107.7	2.8
	5.0	102.6	6.9

Add three concentration levels of atrazine mixed Standard solution to the root blank sample of alfalfa plants to carry out the addition and recovery experiment, and set up five parallel groups for each standard concentration. When the concentration of alfalfa added to the shoot was within the range of 0.05-0.5 mg/kg, the recovery rate was between 83.29% and 101.02%, and the relative standard deviation was relatively between1.29% and 4.32%, which can well meet the requirements of residue detection. This indicated that the accuracy and precision of the experimental method were relatively good, both meeting the requirements of residual analysis (Table 5.5). Previous studies have used ultra-high performance liquid chromatography tandem mass spectrometry to detect residues of benzamide, fipronil, and 2-methyl-4-chloride in milk. It had also been found that there was a good linear relationship between the concentration and peak area of the three pesticides, with high accuracy and good repeatability (Wen et al., 2023).

Table 5.5

The average recovery rate and relative standard deviation of the root methods (PPPI, HAAS, 2023)

Herbicides	Add level (mg/kg)	Recovery (%)	RSD (%)
Atrazine	0.05	83.29	2.77
	0.10	101.02	1.29
	0.50	89.89	4.32

5.5. Detection and analysis of actual samples

The use of UPLC-MS/MS can not only meet the detection requirements of conventional food matrices, but also be suitable for rapid and accurate detection methods of biological samples, and can be applied to qualitative and quantitative

analysis of herbicide residues in large quantities of samples (Vonberg et al., 2014; Hong et al., 2022; Yu et al., 2022). Previous researchers have used this method to detect paraquat pesticide residues in six sample matrices. This method is sensitive, convenient, and versatile (Wang et al., 2022). This experiment measured the absorption of SF and J2 at different periods after treatment by applying atrazine to both SF and J2. The results showed that as time passes, the absorption of atrazine by the shoots and roots of alfalfa continues to increase. In Figure 5.1(A), the absorption of atrazine by the shoots of SF and J2 is faster within seven days after application. The absorption of SF was faster within five days and was in a slow state after seven days. On the 7th day, the absorption amount of J2 was 2.19 times that of SF. Under the same treatment, In Figure 5.1(B), there was a significant difference in the absorption levels of atrazine in the aerial parts of SF and J2. Resistant and sensitive roots were absorbed quickly within three days, and the absorption amount increased slowly after three days. Under the same treatment, the absorption amount of J2 on the third day was 0.97 times that of SF and J2 roots were no significant differences in the absorption levels of atrazine.

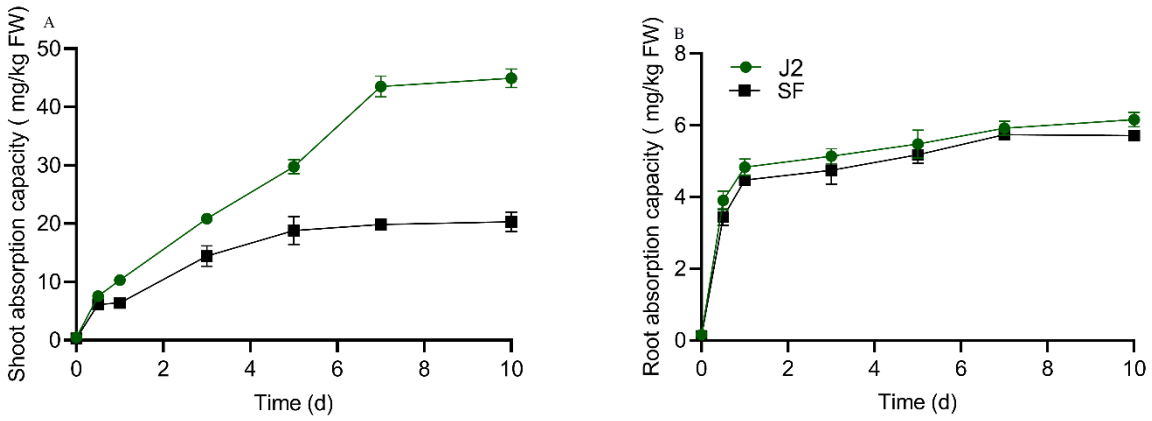

Figure 5.1 Effects of atrazine on shoot (A) and root (B) uptake in SF and J2 alfalfa (PPPI, HAAS, 2023)

After the shoots and roots of SF and J2 were put into the nutrient solution, the resistance to weed in the shoots and roots of SF and J2 was measured at 0, 0.50, 1, 3, 5, 7 and 10 days after treatment. The results showed that both the shoots and root alfalfa metabolized the fastest within three days and metabolized slowly after three days. On the 3rd day, the metabolic amount of J2 was 2.90 times that of the resistant SF. On the

7th day, the metabolic amount of the resistant shoots of alfalfa was 3.35 times that of J2. The metabolic levels of atrazine between SF and J2 showed significant differences. There was no significant difference between the two roots (Figure 5.2).

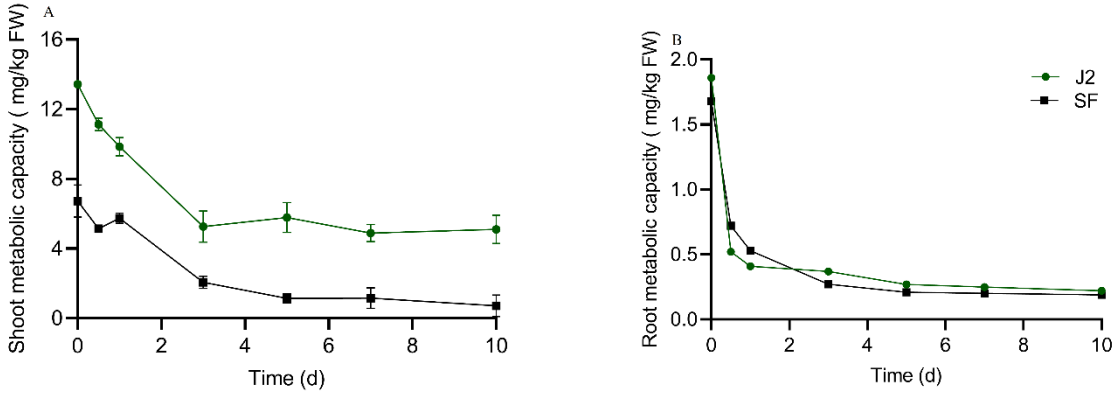

Figure 5.2 Metabolic effects of atrazine on shoots (A) and roots (B) of SF and J2 alfalfa (PPPI, HAAS, 2023)

Atrazine is easily absorbed by plant roots and transmitted upwards, leading to toxic effects when accumulated in plants. It has been reported that the resistance of Poa pratensis to herbicides is due to its enhanced metabolic capacity and reduced absorption and transport of the herbicide. Yasuor also obtained similar results, indicating that the alfalfa resistant may have developed metabolic resistance to herbicide (Yasuor et al., 2019). Iwakami found that the metabolic rate of the resistant variety to atrazine was significantly higher than that of the sensitive alfalfa variety when studying the metabolic resistance of abulan to atrazine (Iwakami et al., 2014). Similarly, Sun discovered that the resistant variety of duckweed also developed metabolic resistance to atrazine (Sun et al., 2021). These experimental results further indicate that the resistance of alfalfa to atrazine was related to absorption metabolism, and alfalfa resistant varieties may have metabolic resistance to atrazine.

Conclusions to Chapter 5

The optimal conditions for the experiment were high sensitivity and good separation of instrument parameters. The parent ion with the best instrument parameters was 217, and the daughter ions were 174 and 68.2, respectively, fragmentor values of 30V and

25V and collision energies of 17eV and 25eV. Retention time was 1.366, and the optimal mobile phase was acetonitrile and 0.1% formic acid water.

In residue detection, the extraction method improved the recovery rate of atrazine. The highest recovery rate was observed when adding 50mg C_{18}+50mg PSA.

Under optimization conditions, the linear equation was y=1541340.7+19861.1, and R=0.9999. Indicating a good linear relationship.

The addition level was within 0.05-5.0 mg/kg, and the recovery rates of alfalfa shoots and roots were measured to be between 83.29% and 107.7%, with a relative standard deviation between 1.29% and 6.9%. This indicated that the method had high accuracy and repeatability.

The resistance of SF and J2 to atrazine was related to their absorption and metabolism of atrazine. In terms of absorption, the residual amount of J2 shoots was always higher than SF. In terms of metabolism, J2 shoot metabolizes atrazine faster than SF, and the difference between the two was significant.

That is based on the results of the fourth chapter, 1 Ukrainian Journal (Zhu & Rozhkova, 2024).

Chapter 6

COMPARATIVE ANALYSIS OF TRANSCRIPTOMES OF RESISTANT AND SENSITIVE ALFALFA VARIETIES UNDER ATRAZINE STRESS

6.1. Sequencing data quality control

The research object of transcriptome sequencing (RNA_Seq) is the sum of all RNA that can be transcribed by a specific cell in a certain functional state, which can comprehensively obtain transcriptome information of specific tissues or organs of a species, and thus conduct research on transcriptome structure, variation, gene expression level, and discovery of new transcripts (Mortazavi et al., 2008). Genomic sequencing technology is widely used in research in medicine, biology, and agriculture, and its application has changed biological research, having a significant impact on crop improvement and optimizing agricultural production models (Wan et al., 2011; Pertea, 2012; Mutz et al., 2013).

In recent years, many scholars have used transcriptome sequencing technology to study the resistance mechanism of plants to herbicides, and thus identified the relevant genes for detoxification metabolism in resistant plants (Saha et al., 2002; Martin & Wang, 2011). Our experiment aims to further explore the reasons for the differences between SF and J2 alfalfa from a molecular biology perspective. A total of 12 samples (4 treatments x 3 replicates) were collected from the aboveground parts of SF and J2 alfalfa after 3 days of application for transcriptome sequencing. Our experiment obtained a total of 553203624 raw reads. The average raw reads for SF-CK, SF-T, J2-CK, and J2-T are 49392117, 46339201, 48520480, and 47094559, respectively. A total of 83.74Gb of clean reads were obtained from the experiment, with an average of 195768447 clean reads for SF-CK, 46339201 clean reads for SF-T, 47078305 clean reads for J2-CK, and 45880988 clean reads for J2-T a total of 83.74Gb of clean bases were obtained from the experiment, with an average clean base of 7.20Gb for SF-CK, 6.77Gb for SF-T, 7.06Gb for J2-CK, and 6.88Gb for J2-T, respectively. All clean bases of the samples reached 6.34 Gb or above, with base error rates less than 0.10%. The Q20 base percentage was above 97.8%, and the Q30 base percentage was above

93.48%, all of which met the requirements for subsequent sequencing (Table 6.1).

In addition, RNA Seq can directly analyze the transcriptome of most organisms, as it does not require knowledge of the genetic information of the target species, thus exhibiting particular advantages. Before the emergence of RNA Seq, people had limited understanding of the transcriptome. RNA Seq has shown both high efficiency and speed, greatly changing people's understanding of the transcriptome. Research has found that by screening key genes involved in specific floral biosynthesis pathways through transcriptome sequencing, and analyzing the differences and reasons for changes in floral aroma among varieties at the gene expression level, sequencing results have high accuracy (Chen et al., 2024). Similarly, Bo found that through transcriptome analysis, multiple important genes and transcription factors involved in heat stress response were identified (Bo et al., 2024). Their experimental results were consistent with ours.

Table 6.1

Sequencing data quality control results **(**PPPI**, HAAS, 2024)**

Sample	Raw reads	Clean reads	Cleans bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
SF-CK-1	50437118	49249400	7.39	0.02	98.12	94.26	42.23
SF-CK-2	48552838	47265968	7.09	0.02	98.26	94.63	42.28
SF-CK-3	49186394	47539972	7.13	0.02	98.13	94.32	42.19
SF-T-1	48870244	47593278	7.14	0.02	98.25	94.64	41.45
SF-T-2	43284816	42245312	6.34	0.03	97.87	93.67	41.42
SF-T-3	46862544	45499158	6.82	0.02	98.26	94.62	41.54
J2-CK-1	52138580	50581258	7.59	0.03	97.96	93.86	42.23
J2-CK-2	45590754	44233492	6.64	0.03	97.8	93.48	42.11
J2-CK-3	47832106	46420166	6.96	0.02	98.32	94.79	42.07
J2-T-1	46204440	44864510	6.73	0.02	98.21	94.46	41.81
J2-T-2	49004928	47830644	7.17	0.02	98.05	94.1	41.83
J2-T-3	46074310	44947810	6.74	0.02	98.15	94.31	41.81

6.2 Differential expression gene analysis

Differentially expressed genes refer to genes that are differentially expressed in two

or more samples under the same conditions, their expression levels are significantly different in different samples. This difference sometimes becomes a change or regulation of gene expression (Jiang et al., 2015; Hrdlickova et al., 2017). The abundance of transcripts reflects the expression level of genes, and the higher the abundance of transcripts, the higher the gene expression level. In RNA Seq analysis, the expression level of genes was calculated by the number of clean reads counts located in genomic regions. Quantitative analysis of gene and transcript expression levels was performed using the RSEM software to facilitate subsequent analysis of differential gene/transcript expression between different samples. By combining sequence functional information, the regulatory mechanisms of genes can be revealed. Expressing quantitative results in units of FPKM, the homogenization process of FPKM ensured that the total expression level is consistent across different samples (Zhang et al., 2020; Liu et al., 2021).

This experiment used DESeq2 software to analyze and compare the differentially expressed genes of J2 and SF alfalfa before and after the application of atrazine (Figure 6.1). The results showed that there were 2479 differential genes between J2-CK and SF-CK before atrazine treatment, of which 1068 genes were up-regulated, and 1411 genes were down-regulated, this illustrated the genetic differences between J2 and SF alfalfa, there were significant differences in levels. After atrazine treatment, there were 4032 differential genes between J2-T and SF-T, of which 2233 were up-regulated and 1799 were down-regulated. Compared with J2-T and J2-CK, there were 30979 differential genes, including 13320 genes. The expression level of SF-T was up-regulated, and the expression level of 17,659 genes was down-regulated. Compared with SF-T, there were 31,181 differential genes, of which 13,907 were up-regulated and 117,274 were down-regulated. Both J2 and SF alfalfa will produce more differential genes after being treated with atrazine. The number of differential genes is the same, but the results were different. J2 eventually dies, while SF alfalfa survived. It may be related to transferases, metabolic enzymes, detoxifying enzymes, etc., but it was still uncertain whether the genetic differences between SF and J2 alfalfa are the

cause of resistance to atrazine.

Differential expression gene screening was one of the important steps in transcriptome analysis. It can screen out genes with significant differences in expression levels, which was very important for studying the biological functions, molecular mechanisms, and biological significance of genes. Previous studies have found that differentially expressed genes in response to cadmium stress in passion fruit leaves are mainly enriched in functional groups such as cell structure, catalytic activity, transcriptional regulation, as well as photosynthesis and carbohydrate metabolism pathways (Zhang et al., 2024). This was similar to our research methodology.

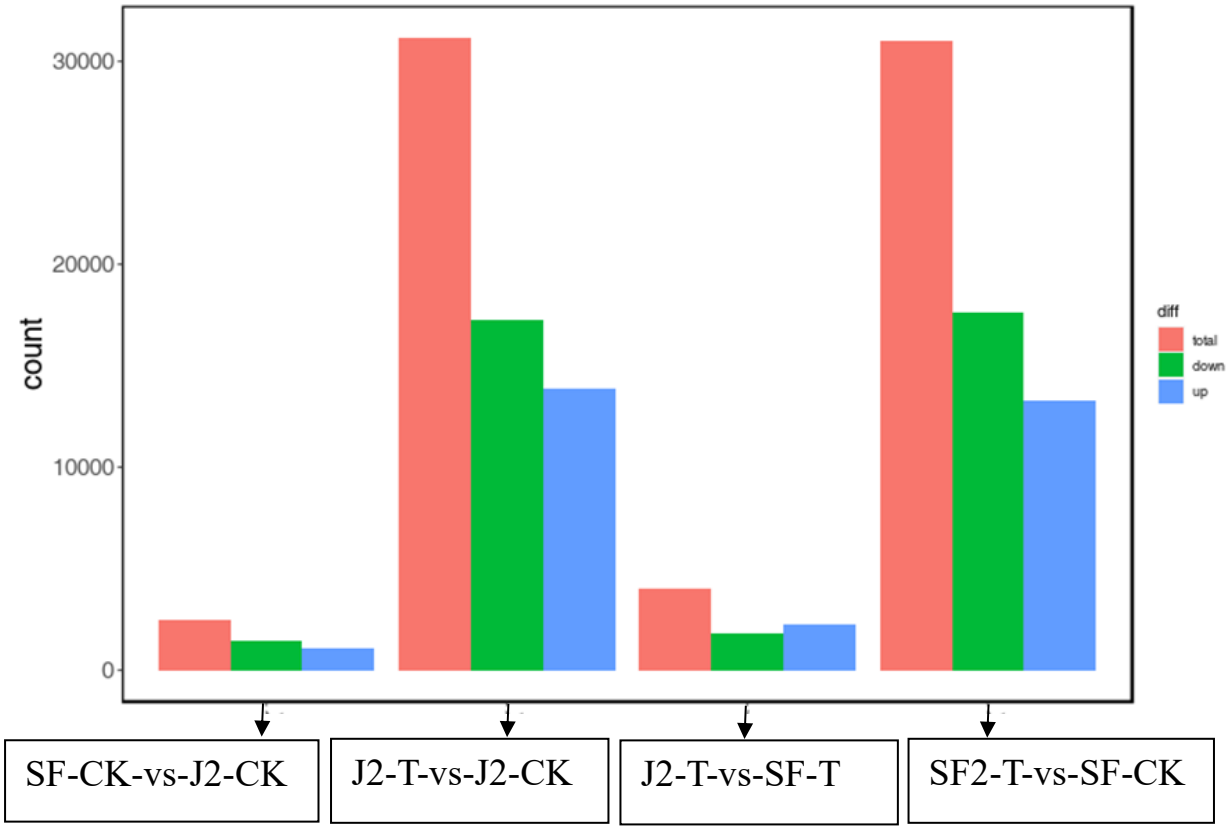

Figure 6.1. Differential gene analysis between SF and J2 alfalfa (PPPI, HAAS, 2024)

6.3 GO enrichment analysis of differentially expressed genes between SF and J2

GO (Gene Ontology) is a database established by the Gene Ontology Consortium with the aim of creating a semantic vocabulary standard that is applicable to various species, limits and describes gene and protein functions, and can be updated as research progresses. It is suitable for all species. By establishing a dynamically structured controlled vocabulary to describe the roles played by genes and proteins within cells,

the properties of genes and gene products in organisms can be comprehensively described (Hu et al., 2021).

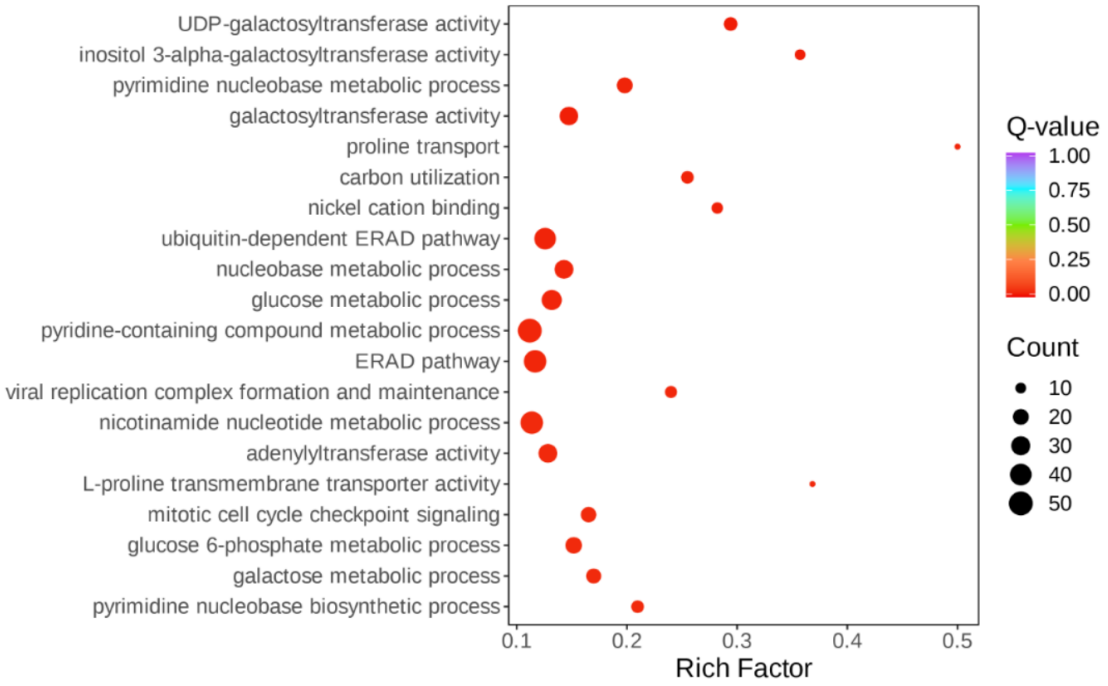

Figure 6.2. GO enrichment of differentially expressed genes in the aboveground parts of alfalfa varieties treated with atrazine in J2 and SF (PPPI, HAAS,2024)

To clarify the biological functions and properties of SF and J2 expression genes under atrazine stress, GO annotation analysis was performed on the differentially expressed genes between herbicide treatment for 3 days and the control group (Table 6.2). Our results showed that the top 10 functions enriched in DEGs were UDP-galactosyltransferase activity, inositol 3-alpha-galactosyltransferase activity, pyrimidine nucleobase metabolic process, galactosyltransferase activity, proline transport, carbon utilization, nickel cation binding, ubiquitin-dependent ERAD pathway, nucleobase metabolic process, glucose metabolic process.

Carbon utilization is a crucial stage in the process of photosynthesis. In the process of carbon utilization, CO_2 is fixed into 3-phosphate glycosides, which are then further synthesized into glucose and other compounds. Non biological stress often damages the photosynthetic apparatus of plants by reducing the rate of photosynthetic electron transfer, inactivating the centers of the pigment system (PS), degrading pigments and proteins, and affecting normal plant growth (Noushina et al., 2021). The results of this

experiment showed that atrazine stress reduced the expression levels of most carbon utilization related genes in photosynthesis. However, compared with the differences in expression levels of PSII related genes, the expression differences of PSII related genes and electron transfer related genes showed a more significant correlation with herbicide resistance. Under atrazine stress, most carbon utilization related genes showed expression differences in J2, while only a small number of genes in the aboveground part of the resistant variety SF were affected by atrazine. This indicated that the differences in resistance of alfalfa were mainly manifested in PSII and electron transfer processes, which was similar to previous studies on the mechanism of herbicide toxicity (Zhang et al., 2017).

UDP galactosyltransferase also played an important role in differential gene expression between SF and J2 in GO enrichment. In recent years, the synthesis of glycoside compounds through enzymatic methods has received increasing attention. At present, the only reported type of sugar modification of marigold glycoside E is glucose modification, especially galactose, which plays an important role in improving the pharmacological activity of natural products as an important sugar modification. By modifying the glucuronic acid at position 3 of soybean soap alcohol B monoglucuronic acid (SBMG) with another galactose, soybean saponin III is formed, which enhances its anti-herpesvirus, anti-hemolysis, and hepatoprotective abilities (Sei et al., 2000). Lactose modified glycoside derivative soy saponin Bb can be used to treat osteoporosis and other conditions. There was a significant difference between SF and J2 genes in this experiment, which may be due to the recognition mechanism of the SF gene when reacting with UDP galactose, preventing herbicide from entering the plant body to avoid drug invasion.

6.4 KEGG enrichment analysis of differentially expressed genes between SF and J2

KEGG pathway enrichment analysis is an important method in bioinformatics, mainly used to reveal the role of genes in specific biological processes. The KEGG database is a database that systematically analyzes the metabolic pathways of gene products in cells and the functions of these gene products. This database helps to study

genes and expression information as a holistic network (Yao et al., 2024). KEGG integrates data on genomics, chemical molecules, and biochemical systems. Including metabolic pathways, drugs, diseases, gene sequences, and genomes. Its main goal was to determine whether the genes observed in the experiment are concentrated in specific functions or pathways, in order to infer whether these functions or pathways are significantly enriched under experimental conditions. It is also possible to choose one's favorite gene set for research questions and experimental design.

To analyze the metabolic regulatory pathways of differentially expressed genes in SF and J2 alfalfa under atrazine stress, KEGG pathway analysis was performed on the differentially expressed genes between SF and J2 alfalfa treated with atrazine for 3 days and the control (Figure 6.3). The results showed that the top 10 enriched pathways were compared among all treatments. The pathways enriched by DEGs in the aboveground parts of SF and J2 mainly include ribosomes, carbon metabolism, galactose metabolism, various types of n-glycan biosynthesis, biosynthesis of amino acids, RNA polymerase, and mRNA surveillance pathway.

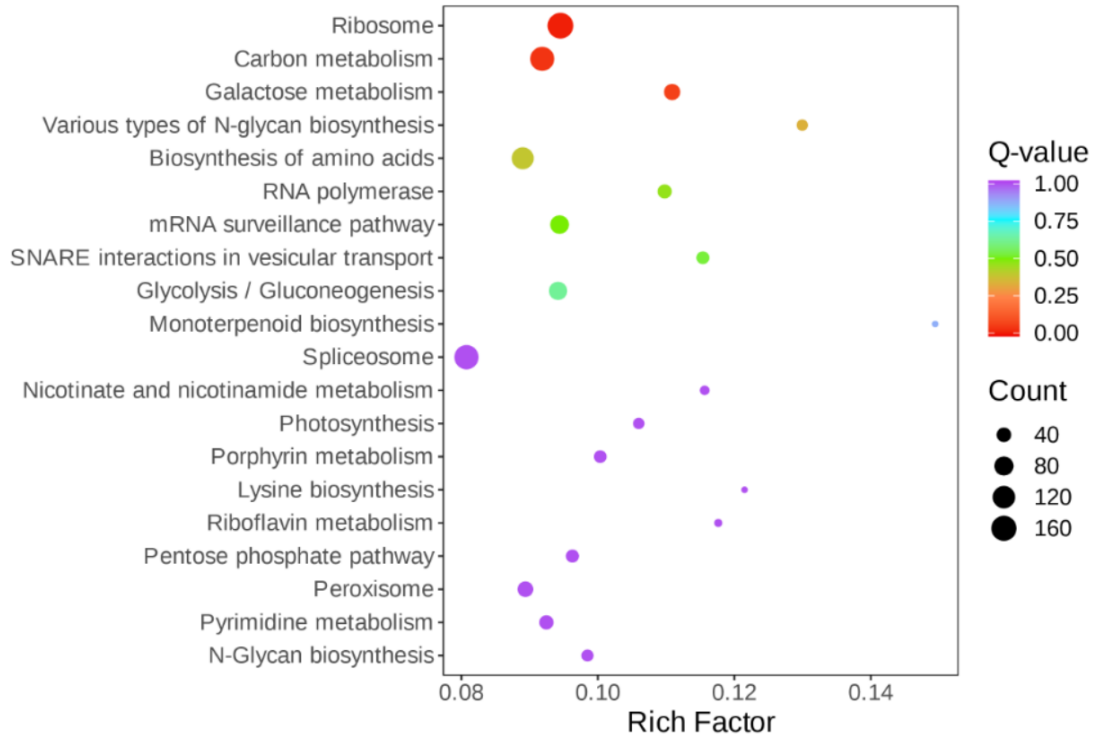

Figure 6.3. KEGG enrichment of differentially expressed genes in the aboveground parts of alfalfa varieties treated with atrazine in J2 and SF (PPPI, HAAS, 2024)

Among them, carbon metabolism is a general term for a series of physiological and biochemical processes in which plants assimilate inorganic carbon dioxide into organic carbohydrates during photosynthesis, and transform organic carbon into carbon dioxide during respiration and photorespiration (Ren et al., 2023). This included the synthesis, degradation, and transformation of photosynthetic products such as starch and sucrose, as well as glycolysis, tricarboxylic acid cycle, pentose phosphate pathway, glycolic acid oxidation pathway, and glyoxylate cycle during respiration. Carbon metabolism is the fundamental metabolism for the growth and development of alfalfa, which affects plant growth and yield formation, as well as the regulation of carbon metabolism enzymes (Saengtharatip et al., 2021). In this experiment, there was a significant difference between SF and J2 alfalfa under atrazine stress, which may be due to a series of physiological and biochemical reactions of J2 under photosynthesis and respiration. Yang found that low temperature stress affects the metabolic pathways of carbohydrates in upright mung beans (Wan et al., 2024). This result was basically consistent with the results of this experiment.

Amino acids are important organic substances in living organisms. They are the basic building blocks of proteins and important substances for maintaining life activities. The synthesis of amino acids mainly occurs in plants, which can be divided into two processes: photosynthesis and non-photosynthesis (Fu et al., 2020). Photosynthesis is the main process of amino acid synthesis in plants. It is the process in which plants use solar energy to convert carbon dioxide and water into organic matter, and amino acids are also one of them. During photosynthesis, plants use solar energy to convert carbon dioxide and water into organic matter, including amino acids (Fang et al., 2021). In this experiment, there was a significant difference between SF and J2 alfalfa under atrazine stress, which may be due to the response of J2 alfalfa in photosynthesis, leading to the destruction of J2 alfalfa, while SF showed no obvious symptoms. Huang found that there are differences in the content of amino acids, especially theanine, among different tea tree varieties when studying the progress of amino acid synthesis, metabolism, and transformation in tea. The main difference is

that the theanine content in yellowing or whitening varieties is higher than that in green leaf varieties (Huang et al., 2023). Liu compared the gene expression levels of theanine metabolism pathways and found that the expression levels of CsTS2, CsGS1, and CsGDH2 genes were positively correlated with the content of theanine among varieties (Liu et al., 2017). Cheng demonstrated through stable isotope tracing experiments that weak hydrolysis ability of theanine may lead to the accumulation of high theanine in yellowing or whitening varieties (Cheng et al., 2018). These research results were basically consistent with our experimental KEGG enrichment analysis results.

6.5 Analysis of gene expression levels of SF and J2 key pathways

In order to investigate the relationship between DEGs and resistance of SF and J2 alfalfa under atrazine stress, we combined GO and KEGG enrichment analysis results with previous studies on plant stress response to screen for pathways that may be related to variety differences in atrazine resistance in alfalfa, including photosynthetic carbon utilization and metabolism, amino acid synthesis and UDP galactosyltransferase.

6.5.1. Photosynthetic carbon utilization and metabolism

The photosystem was two multi subunit basement membrane protein complexes involved in carbon photosynthesis. Under the stress of atrazine, the expression levels of most photosynthesis related genes were reduced (Table 6.2). Compared with the carbon metabolism expression level of SF, J2 showed downregulated expression of Log2FC in genes MS.gene 012982, MS.gene 013827, MS.gene 059022, MS.gene 75432, and MS.gene 95519, with values of -0.7375, -1.2614, -1.9312 and -1.9312. While in MS.gene 038054 and MS.gene 043136, the values of Log2 FC were 2.1308, 2.130 and 2.130, indicating upregulation of expression in these genes.

The expression differences of PSII related genes and electron transfer related genes showed a more significant correlation with herbicide resistance, with more pronounced expression differences occurring in J2 under herbicide stress and more pronounced under herbicide stress. This indicated that the differences in resistance of alfalfa were mainly manifested in PSII and electron transfer processes, which was similar to

previous studies on the mechanism of herbicide toxicity (Zhu et al., 2022).

Table 6.2

Differential expression of carbon utilization and metabolism related genes in photosynthesis between SF and J2 (PPPI, HAAS, 2024)

Gene name	Gene ID	Log2 FC Shoot (SF vs J2)
Carbon utilization and metabolism	MS.gene 012982	-0.7375
Carbon utilization and metabolism	MS.gene 013827	-1.2614
Carbon utilization and metabolism	MS.gene 059022	-1.9312
Carbon utilization and metabolism	MS.gene 75432	-1.9312
Carbon utilization and metabolism	MS.gene 95519	2.1308
Carbon utilization and metabolism	MS.gene 038054	2.130
Carbon utilization and metabolism	MS.gene 043136	2.130

6.5.2. Amino acid synthesis

The synthesis and metabolism of amino acids are the cornerstone of small molecule and protein metabolism in plants, and are important components of plant nutrition (Xiong et al., 2023). Atrazine stress increased the expression levels of most acid synthesis related genes (Table 6.3). Compared with the expression levels of amino acid synthesis in J2, the Log2 FC values of SF in genes MS.gene 029341, MS.gene 073220, MS.gene 08146, MS.gene 29716, MS.gene 37166, MS.gene 94185, MS.gene 99608, MS.gene 039984, MS.gene 049265, MS.gene 41792, and MS.gene 84528 were 1.0282, 0.9591, 0.9596, 2.3637, 2.5484, 1.9306, 0.9571, 1.0233, 0.5975, 0.9385, and 1.0782. These genes showed upregulation of SF gene expression under herbicide stress. In MS.gene 23053, MS.gene 64377, MS.gene 96839, MS.gene 08062, MS.gene 34073, and MS.gene 84523, the Log2 FC values were -1.1987, -0.9216, -0.7773, -6.1542, -2.9831 and -2.3637. These genes showed downregulation of SF gene expression under herbicide stress.

There were differences in the metabolism and accumulation of amino acids between SF and J2 alfalfa. Research has found that when studying the synthesis and metabolism of sulfur-containing amino acids in soybeans, Fan discovered that the first key enzyme

gene in the amino acid synthesis pathway catalyzes the synthesis of O-acetylserine from serine, regulating the metabolic dynamic balance in plants (Fan et al., 2023). Zhang cloned and identified six genes, including GmOAS-TL1, GmOAS-TL2, GmOAS-TL3, GmOAS-TL4, GmOAS-TL6, and GmOAS-TL7, in cultivated soybeans. Among them, four genes, GmOAS-TL1, GmOAS-TL3, GmOAS-TL4, and GmOAS-TL6, can catalyze the synthesis of cys. These genes show differences in expression levels in different tissues and developmental stages, suggesting that the synthesis of cys in soybeans may be regulated by multiple OAS-TLs (Zhang et al., 2008). This result confirmed that in studying the differential genes between SF and J2 alfalfa, amino acid synthesis can help SF maintain internal balance and normal growth and development under herbicide stress.

Table 6.3

Differential expression of amino acid synthesis related genes SF and J2 (PPPI, HAAS, 2024)

Gene name	Gene ID	Log2 FC Shoot (SF vs J2)
Amino acid synthesis	MS.gene 029341	1.0282
Amino acid synthesis	MS.gene 073220	0.9591
Amino acid synthesis	MS.gene 08146	0.9596
Amino acid synthesis	MS.gene 23053	-1.1987
Amino acid synthesis	MS.gene 29716	2.3637
Amino acid synthesis	MS.gene 37166	2.5484
Amino acid synthesis	MS.gene 64377	-0.9216
Amino acid synthesis	MS.gene 94185	1.9306
Amino acid synthesis	MS.gene 96839	-0.7773
Amino acid synthesis	MS.gene 99608	0.9571
Amino acid synthesis	MS.gene 039984	1.0233
Amino acid synthesis	MS.gene 049265	0.5975
Amino acid synthesis	MS.gene 08062	-6.1542
Amino acid synthesis	MS.gene 34073	-2.9831

Amino acid synthesis	MS.gene 41792	0.9385
Amino acid synthesis	MS.gene 84523	-2.3637
Amino acid synthesis	MS.gene 84528	1.0782

6.5.3. UDP galactosyltransferase

Glycosylation reaction is one of the important transformation reactions in living organisms. Glycosyltransferases are enzymes specifically responsible for catalyzing glycosylation reactions, with strong substrate specificity, and are one of the most diverse enzymes in nature. Glycosylation reaction is one of the important transformation reactions in living organisms (Sun et al., 2024). UDP galactosyltransferase was one of the most extensively studied glycosyltransferases in recent years. Our experiment found that herbicide stress increased the expression levels of most UDP galactosyltransferase related genes (Table 6.4). Compared with the amino acid synthesis expression level of J2, the Log2 FC values of SF in genes MS.gene 96146, MS.gene 29304, and MS.gene 78327 were 2.8976, 1.1254 and 0.9758, respectively. These genes showed upregulation of SF gene expression under herbicide stress, while the Log2 FC value in MS.gene 072436 was -1.7465, indicating downregulation of SF gene expression under herbicide stress.

There were differences in UDP galactosyltransferase expression between SF and J2 genes. At present, the only reported type of sugar modification of marigold glycoside E is glucose modification, especially galactose, which played an important role in improving the pharmacological activity of natural products as an important sugar modification. Functional validation of soybean glycosyltransferase GmSGT2 catalyzing the reaction of marigold glycoside E with UDP galactose was conducted, and the recognition mechanism of GmSGT2 catalyzing the reaction of marigold glycoside E with UDP galactose was revealed through molecular docking studies (Gao et al., 2020). The UDP galactosyltransferase studied in this result was functionally consistent with the present study in SF.

Table 6.4

Differential expression of UDP galactosyltransferase related genes SF and

J2(PPPI, HAAS, 2024)

Gene name	Gene ID	Log2 FC Shoot (SF vsJ2)
UDP galactosyltransferase	MS.gene 07246	-1.7465
UDP galactosyltransferase	MS.gene 96146	2.8976
UDP galactosyltransferase	MS.gene 29304	1.1254
UDP galactosyltransferase	MS.gene 78327	0.9758

Conclusions to Chapter 6

Verified the reliability of transcriptome sequencing data from a molecular biology perspective. The clean bases of each sample reached 6.34 Gb or above, with base error rates less than 0.10%. The Q20 base percentage was above 97.8%, and the Q30 base percentage was above 93.48%, indicating that the sequencing data met the requirements.

Under the stress of atrazine, SF and J2 produced a significant number of differentially expressed genes. There were differences in phenotype and genes between the two.

The GO enrichment analysis of SF and J2 differentially expressed genes mainly focused on UDP galactosyltransferase activity, galactosyltransferase activity, and carbon utilization. The difference between SF and J2 may be related to them.

The KEGG enrichment analysis of SF and J2 differentially expressed genes mainly focused on biosynthesis of amino acids and carbon metabolism. The difference between SF and J2 may be related to them.

We identified SF and J2 key pathway genes primarily involved in photosynthetic carbon utilization and metabolism, amino acid synthesis, and UDP galactosyltransferase, these genes were one of the main reasons for SF and J2 to develop resistance to atrazine.

CHAPTER 7

THE FIELD APPLICATION OF ATRAZINE ON ALFALFA PLANTS

7.1. Atrazine residues in soil of resistant SF and sensitive J2 alfalfa

Atrazine is widely used as herbicide, which not only increases crop yield, but also pollutes soil, groundwater, Rivers and Lakes and other environments. Although atrazine has low toxicity, it is easily soluble in water, difficult to degrade, and has a large residue, which affects crop yield and hinders the sustainable development of agriculture in China. In particular, the biological cycle can allow residual harmful substances to enter the human body, posing a great threat to humans (Krutz et al., 2009). There have been no reports on the residue and degradation dynamics of atrazine in alfalfa plants and soil. This experiment was conducted in Xinxiang City, Henan Province, China, from 2022 to 2024 to study the residue and safe use technology of atrazine in alfalfa. This provided a technical basis for the scientific and rational use and limits research of atrazine in alfalfa. In preliminary pot experiments, we found that atrazine has toxic effects on resistant SF and sensitive J2. Many reports indicated that atrazine behaves differently on pot plants and the field. In the two-year experiments of cultivation in 2022-2023 year and 2023-2024 year, the residue of atrazine in the soil showed a decreasing degradation trend. In 2023, on the 120th day, the degradation rate of atrazine in soil reached 54.49%, the degradation rate reached equilibrium on the 120-180 day, and on the 180th day, the degradation rate of atrazine in soil reached 84.44%. In 2024, on the 150th day, the degradation rate of atrazine in soil reached 48.31%, reaching equilibrium on the 150-180 day, and on the 180th day, the degradation rate of atrazine in soil reached 66.67% (Table 7.1).

The structure of atrazine is very stable, and its retention time in soil varies with soil type. However, it can be confirmed that microbial mineralization occurs very slowly on atrazine, making it difficult to degrade (Song et al., 2022). This experiment found that after spraying atrazine, the residual amount in the soil gradually decreased with the prolongation of time, but it persisted, which is consistent with Zhao's experimental results (Zhao et al., 2024). In the environment, for atrazine, the role of soil was that of

a warehouse and a station. Over time, the harm of atrazine in soil may be transferred or delayed, but potential risks still exist. To completely solve the residue of atrazine, degradation was necessary. Many non-governmental organizations and academia have now nominated atrazine as a new persistent organic pollutant substance.

Table 7.1

Degradation dynamics of atrazine in field soil

(PPPI, HAAS, 2023)

Days	2023	2024
	Soil residue (mg/kg)	Soil residue (mg/kg)
0	15.00±0.00	16.00±0.00
7	11.97±0.54	13.97±0.54
21	9.08±0.23	12.08±0.96
42	7.71±0.34	7.71±0.62
60	6.83±0.18	9.83±0.18
120	5.27±0.21	8.27±0.57
150	3.24±0.38	5.24±0.20
180	2.33±0.01	5.33±0.99

Note: Data expressed as mean ± standard error, lowercase letters indicate the significance of the difference tested by DPS (Duncan, $p<0.05$).

7.2 Residue of atrazine in resistant SF and sensitive J2 alfalfa plants

Atrazine not only has strong killing power against target weeds, but also has a long residual effect time and is not easily degraded, causing hidden phytotoxicity to subsequent crops such as rice, wheat, soybeans, and sugar beets. Field experiments have found that after planting vegetables instead of corn for a long time, the phenomenon of dead seedlings often occurs, this is caused by the long-term use of atrazine in the previous corn field (Ye et al., 2001).

In our two-year cultivation trials of 2022-2023 and 2023-2024, the residual levels

of atrazine in SF and J2 plants showed an initial increase followed by a decreasing trend. In the 2023 experiment, on the 120th day, the residual amount of atrazine in SF reached 11.32 mg/kg, and the residual amount in J2 reached 23.29 mg/kg. At this time, the residual amount of atrazine in SF and J2 reached its maximum value, and there was a significant difference in the residual amount between SF and J2. On the 180th day, the residual levels of atrazine in SF and J2 showed a decreasing trend, with the residual levels in SF dropping to 6.23 mg/kg and J2 dropping to 10.21 mg/kg, respectively. Moreover, there was still a significant difference in residual levels between SF and J2 plants (Table 7.2).

In the 2024 experiment, on the 120th day, the residual amount of atrazine in SF reached 15.03 mg/kg, and the residual amount in J2 reached 25.29 mg/kg. At this time, the residual amount of atrazine in SF and J2 reached the maximum value, and there was a significant difference in the residual amount between SF and J2 plants. The residual amount of J2 was 1.68 times that of resistant SF. On the 180th day, the residual levels of atrazine in SF and J2 showed a decreasing trend. At this time, the residual levels in SF decreased to 7.23 mg/kg, and J2 decreased to 13.31 mg/kg, and there was still a significant difference in the residual levels between SF and J2 plants. The residual level in sensitive J2 plants was 1.84 times higher than in resistant SF plants (Table 7.2).

Numerous studies have shown that the residue of atrazine in soil can seriously affect the growth and development of subsequent crops. Dai also observed that the longer the exposure time and the higher the concentration of high-efficiency cypermethrin residue on alfalfa phytotoxicity symptoms, the deeper the degree of phytotoxicity (Dai et al., 2014). Shen also found in their study on the phytotoxicity of organophosphate to tobacco that as the concentration of organophosphate application increased, the inhibition rates of alfalfa plant height, leaf length, and leaf width also increased. Alfalfa showed significant dwarfism, reduced leaf area, and decreased yield per plant (Wang et al., 2012). It can be seen that there is a significant dose-response relationship between the phytotoxicity of atrazine on alfalfa and its application dose.

Table 7.2

Residual levels of atrazine in SF and J2 plants in the field (PPPI, HAAS, 2024)

Days	2023		2024	
	SF residue (mg/kg)	J2 residue (mg/kg)	SF residue (mg/kg)	J2 residue (mg/kg)
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
7	0.60±0.15	2.56±0.73	1.60±0.44	2.56±0.45
21	1.22±0.12	3.56±0.27	2.22±0.12	3.56±0.90
42	4.03±0.46	6.21±0.37	5.03±0.12	7.21±0.42
60	5.01±0.27	13.01±1.39	6.01±0.95	15.01±0.85
120	12.03±0.85	23.29±2.57	15.03±1.36	25.29±1.58
150	9.47±0.63	16.01±0.63	12.47±0.63	19.01±0.46
180	6.23±0.20	10.31±2.01	7.23±0.84	13.31±1.55

Note: Data expressed as mean ± standard error, lowercase letters indicate the significance of the difference tested by DPS (Duncan, $p<0.05$).

Conclusions to Chapter 7

In field experiment, atrazine remained in the soil and harmed subsequent crops. During the periods of 2022-2023 and 2023-2024, as the number of days of residual application of atrazine increased, the residue of atrazine in the soil showed a trend of first decreasing and then stabilizing.

Residual atrazine was detected in SF and J2 alfalfa, it posed a threat to their growth. During the periods of 2022-2023 and 2023-2024, as the number of days of residual application of atrazine increased, the residual amount of atrazine in SF and J2 showed a trend of first increasing and then decreasing, and the difference in residual amount between the two was significant.

That is based on the results of the sixth and seventh chapters, 1 Conference papers was published (Zhu & Rozhkova, 2024).

CONCLUSION

The dissertation study studied the harmfulness of atrazine and the mechanisms of alfalfa resistance to this herbicide, which will allow for the eco-friendly protection of corn and improve the quality of feed products.

1. Under the stress of atrazine, the fresh weight inhibition rates of 60 alfalfa varieties were different, indicating that there were differences in the sensitivity of different alfalfa varieties to atrazine.

2. Under the stress of atrazine, SF with the highest IC_{50} and J2 alfalfa with the lowest were selected, indicating that SF developed the highest resistance to atrazine and J2 was the most sensitive to atrazine.

3. By comparing the synergistic effects of malathion and NBD-CI on atrazine in different alfalfa varieties, it was shown that P450s were involved in the development of resistance to SF metabolism, while NBD-CI was not involved. From this, it can be seen that the resistance of SF and J2 to atrazine was related to P450s and not to GSTs.

4. SF and J2 both suffered varying degrees of damage under atrazine stress, but the differences between the two were significant. The PH, RH, SDW and RDW of SF and J2 were all damaged. The indicated differences in resistance between SF and J2 to atrazine.

5. Under the stress of atrazine, the Pn of SF and J2 alfalfa, Gs and Tr decreased, while Ci increased, and the difference between the two was significant. Alfalfa reduced the damage of atrazine to its leaf photosynthetic system by enhancing respiration.

6. Under the stress of atrazine, the Fv/Fm, Fv/Fo, Y (II), ETR, and qP of SF and J2 decreased, while NPQ increased, and the difference between the two was significant. This indicated that atrazine stress caused damage to the PSII reaction centers of SF and J2, thereby inhibiting plant photosynthesis.

7. The residue of atrazine reduced the content of chlorophyll a and chlorophyll b in SF and J2 alfalfa leaves, and the difference between the two was significant. J2 was more sensitive to atrazine than SF.

8. Atrazine residue increased the MDA content in SF and J2 alfalfa and the

significant difference between the two indicated that J2 was more susceptible to damage under atrazine stress than SF.

9. The absorption and metabolic residue detection of SF and J2 by UPLC-MS/MS showed that compared to SF, J2 had faster absorption but slower metabolism, and the difference between the two was significant. SF was more resistant to atrazine than J2 alfalfa.

10. Verified the reliability of transcriptome sequencing data from a molecular biology perspective. The clean bases of each sample reached 6.34 Gb or above, with base error rates less than 0.10%. The Q20 base percentage was above 97.8%, and the Q30 base percentage was above 93.48%, indicating that the sequencing data met the requirements.

11. Through GO and KEGG analysis, key pathway genes involved in photosynthetic carbon utilization and metabolism, amino acid synthesis, and UDP galactosyltransferase activity in SF and J2 were identified, indicating that these genes were one of the main reasons for SF and J2 to produce anti atrazine.

12. In field experiments conducted between 2022-2023 and 2023-2024, the residue of atrazine in soil showed a trend of first decreasing and then stabilizing, while the residue in SF and J2 showed a trend of first increasing and then decreasing, with significant differences. It indicated that atrazine will remain in the soil and alfalfa.

RECOMMENDATION FOR PRODUCTION

An atrazine-sensitive alfalfa variety, Juneng 2, has been identified, which is able to actively absorb this herbicide from the soil according to our research. We suggest using this variety for phytoremediation.

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Figure 1. Experiment on planting SF and J2 alfalfa in the field

Figure 2. Experiment on cultivating 60 alfalfa varieties in artificial greenhouse

Figure 3. Experimental study on residual detection using UPLC-MS/MS

Figure 4. Experimental detection of physiological and biochemical indicators for SF and J2 alfalfa

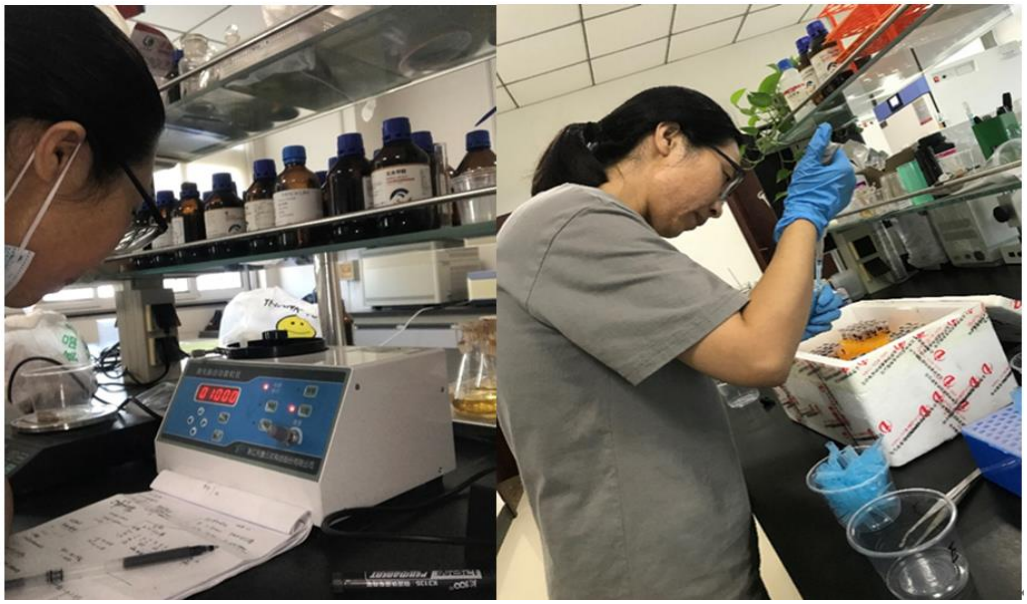

Figure 5. Transcriptome sequencing experiments on SF and J2 alfalfa

"ЗАТВЕРДЖУЮ"
Проректор з науково-педагогічної
та навчальної роботи,
доктор економічних наук, професор
_____ Маргарита ЛИШЕНКО
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АКТ
про впровадження результатів дисертаційної роботи
в навчальний процес

Даним актом стверджується, що результати дисертаційної роботи Чжу Іньхуей на тему «Insights into profiling of resistance mechanism of alfalfa to atrazine» («Профілювання механізму стійкості люцерни до атразину») на здобуття наукового ступеня доктора філософії з галузі знань 20 Аграрні науки та продовольство за спеціальністю 202 Захист і карантин рослин впроваджені у навчальний процес під час викладання дисциплін «Основи агротоксикології» (СВО Молодший бакалавр), «Шкідлива рослинність та карантинні види» та «Імунітет рослин» (СВО Бакалавр), «Токсикологія пестицидів» та «Управління чисельністю бур'янів в агрофітоценозах» (СВО Магістр)

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