UDC 632.95: 633.31

DETERMINATION OF ALFALFA RESISTANCE TO ATRAZINE

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With the rapid development of animal husbandry, finding efficient and sustainable feed resources has become an important issue for the industry's development. Alfalfa is rich in nutrients and has the characteristics of rapid growth and good palatability, which has attracted the attention of many dairy farmers. Atrazine is one of the commonly used herbicides in corn crop, and corn-alfalfa rotation is a common planting mode in the world. However, alfalfa is extremely sensitive to herbicides, so it is crucial to study the resistance of alfalfa to them. Indoor bioassay is a commonly used method for identifying herbicide resistance in crops, which has been widely applied in the study of tolerance to herbicides such as bensulfuron methyl in crops such as alfalfa and soybean. However, there are few reports on indoor bioassay methods for herbicide resistance in alfalfa and related studies on herbicide tolerance in alfalfa. The purpose of this study is to analyze the fresh weight of 60 alfalfa varieties under herbicide stress, to screen resistant and sensitive alfalfa varieties to herbicide influence, to determine the resistance level of alfalfa varieties to herbicide, and to provide theoretical reference for a deeper understanding of herbicide resistance mechanisms and variety improvement in alfalfa. This experiment used the potted soil toxicity method to determine the resistance of 60 alfalfa varieties. The pre experiment concentrations were set as follows: 5.00, 10.00, 20.00, 40.00, 80.00, and 160.00 mg/kg (for resistance determination) and 0.025, 0.05, 0.10, 0.20, 0.40, and 0.80 mg/kg (for sensitivity determination. The most resistant variety (SF8001) and the most sensitive variety (Juneng 2) to atrazine were screened. Among them, the IC_{50} of SF8001 was 14.875, and the IC_{50} of Juneng 2 was 0.2428. Compared with the variety Juneng 2, the resistance multiple (RI) of SF8001 was 61.26. The experimental results indicate that residual herbicides can have toxic effects on alfalfa, and different types of alfalfa have different resistance to herbicides. This provides a theoretical basis for the study of resistance mechanisms and a scientific basis for the selection of alfalfa varieties for rotation planting in corn crop.

Key words: *atrazine, alfalfa, toxicity, pesticide resistance.*

DOI https://doi.org/10.32782/agrobio.2024.3.2

Introduction. Atrazine [2-Chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] is a triazine herbicide (Armstrong et al., 1967; Barriuso et al., 1994; Vonberg et al., 2014a). Since it was developed by Syngenta in Switzerland in the 1950s, it has been widely used in corn, sorghum (*Sorghum bicolor* L. Moench), sugarcane (*Saccharum officinarum* L.) and other planting areas to control annual weeds and broadleaf weeds due to its easy production, wide spectrum of weed control and low cost. It has rapidly developed into one of the most widely used pesticide varieties and has long occupied an important position (Cooper et al., 2007; Cooper et al., 2000; Cox, 2001). It's main mechanism of action is to compete with plastoquinone for the binding site of the photosynthesis center on the thylakoid membrane, block the electron transport chain between the electron acceptor protein and plastoquinone, specifically interfere with photosystem II (PS II), reduce the efficiency of the CO_2 fixation process, and thus affect the normal growth and development of the target weeds (Lazorko-Connon & Achari, 2009; LeBlanc & Sleno, 2011; Piletsky et al., 1995). However, atrazine has a long half-life in the environment, about 57 weeks, and its residual capacity far exceeds its natural degradation capacity. Therefore, atrazine often remains in the soil for about a year, which can easily cause toxic effects on subsequent crops (Ribaudo & Bouzaher. 1994; Roeth et al., 1969; Shimabukuro et al., 1969). Studies have shown that atrazine has a significant inhibitory effect on the plant height and fresh weight of cucumbers (Robinson, 2008). Under atrazine stress, the germination potential, germination rate, root length and bud length of millet are significantly inhibited. It has been found in previous studies that atrazine affects watermelon seed germination and it can significantly inhibit the number of its lateral roots (Sher et al., 2021). Metzger et al. found the concentration of atrazine reached 0.75 g/L, the corn varieties Ludan 981, Yuyu 26 and Zhongdan 5384 growth was significantly inhibited (Metzger et al., 2019). Despite this, in the absence of ideal alternatives, atrazine or herbicides containing atrazine as the main component are still the most widely used herbicides in corn fields and will continue to be used on a large scale for a long time in the future.

Alfalfa (*Medicago sativa* L.), a perennial legume crop, has important ecological and economic value and is increasingly valued in production. In recent years, the area of alfalfa cultivation in China has continued to expand (Castonguay et al., 2006; Zhang et al., 2019). Through the introduction of excellent varieties and the continuous improvement of planting techniques, the yield and quality of alfalfa have been improved, playing an important role in improving the ecological environment and alleviating the shortage of high-quality forage in China (Zhang et al., 2019; Zhang et al., 2014; Zhu et al., 2021). With the development of legume crops, the rotation planting model of corn and alfalfa has been adopted by more and more regions.

Atrazine is one of the common herbicides in corn fields, but the amount of herbicides that can actually act on weeds is very small, and most of them remain in the soil, which has a serious impact on subsequent crops (Zhang et al., 2014; Zhu et al., 2019). Currently, in corn fields of Henan Province (China) excessive atrazine residues it in the soil will continue to grow, and the degree of damage to subsequent crops has become more obvious, seriously affecting the adjustment of planting structure (Yu et al., 2017).

The Alfalfa is a target plant for the herbicide atrazine because it does not contain glutathione transferase and cannot detoxify. It is generally sensitive to the toxicity of atrazine and is very likely to cause a reduction in yield or even a complete failure under atrazine toxicity (Vonberg et al., 2014b; Wackett et al., 2002; Wang et al., 2005). Therefore, reducing the harm of soil residual atrazine to alfalfa production has become an important problem facing large-scale alfalfa production. Screening of alfalfa germplasm for atrazine resistance is an important direction for discovering atrazine resistance genes in alfalfa and developing new resistant alfalfa varieties. At present, there are few reports on the effects of atrazine residues on alfalfa germination. The purpose of this experiment is to identify and evaluate the growth indicators of alfalfa varieties, to screen varieties with strong atrazine resistance as well as sensitive ones, and to provide a reference for alfalfa atrazine-resistant varieties breeding and to study of resistance mechanisms.

Materials and methods. *Seed treatment.* Alfalfa seeds were provided by Henan Academy of Agricultural Sciences. Uniform alfalfa seeds were selected, soaked in warm water at about 30°C for 10 min, then rinsed with distilled water, placed in an incubator at 25°C and soaked for 12 h, then spread flat on a culture dish lined with wet gauze, and cultured in an incubator in the dark until the seeds turned white for later use.

Experimental design. Screening of 60 alfalfa varieties for resistance to atrazine. The indoor poison soil method was tested according to Su's method. and the germination treatment was tested according to Wu's method (Su et al., 2016; Sun et al., 2019). The soil used was taken from the surface layer (0-10 cm) of Yuanyang base area of Henan Academy of Agricultural Sciences. 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. The medicated soil was prepared by soil addition method: 200 g of air-dried sieved soil was placed in a plastic pot (70 mm high × 83 mm in diameter); 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. Pure water was set as the treatment control. The soil was completely moistened by bottom infiltration, balanced overnight, and mixed thoroughly to prepare medicated soil of different concentrations. The sensitivity of alfalfa was initially determined, and the fresh weight inhibition rate of 60 alfalfa species was evaluated after 6 weeks (42 days) of treatment.

Determination of resistance levels of 13 alfalfa varieties to atrazine. Thirteen alfalfa varieties suspected to be sensitive or tolerant to atrazine were screened for further toxicity testing. (The soil concentrations of atrazine in the pre-test were set as follows: resistant – 5.0, 10.0, 20.0, 40.0, 80.0 and 160.0 mg/kg, sensitive – 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/kg, with water as the control. Bottom infiltration was used to make the soil completely wet. After germination whitened uniform seeds were sown in plastic pots, (8 seeds per pot) with 3 replicates per treatment. Then they were placed in an artificial climate chamber with a photoperiod of 12h, light intensity of 22 000 lx, culture temperature of 25-28 ^{o}C, and relative humidity of 75%-80%. Water was weighed and added each day during the culture period to ensure soil moisture. After 42 days of cultivation, the aboveground part of alfalfa was cut, the fresh weight was measured, and the fresh weight inhibition rate as well as 50% inhibition concentration (IC_{50}) were calculated. The sensitivity of different alfalfa varieties to atrazine residues was determined based on the IC_{50} value.

Statistical analysis. All experimental data were calculated, statistically analyzed and drawn using Microsoft Excel 2003, DPS v7.05, GraphPad Prism 9.0 and other software. Data were expressed as mean ± standard deviation, One-WayANOVA analysis was performed using DPS v7.05, and Duncan's new multiple range method was used to compare results and analyze significant indicators in variance analysis.

Results. *1. Screening of atrazine resistance in 60 alfalfa varieties.* The inhibitory effect of 0.2 mg/kg and 10 mg/kg atrazine on the growth of 60 alfalfa varieties was determined by indoor soil toxicity method. The results showed that the alfalfa varieties of XL, YK, Juneng 2, J601, and 801 were more sensitive to atrazine, and the fresh weight inhibition rate of alfalfa growth was more than 70% at 0.2 mg/kg of atrazine, while the varieties of FL, HG, 9720s, SD10, ZM3, WL168HQ, YS, 8421s, SF8001, and J301 were more resistant to alfalfa, and the fresh weight inhibition rate of alfalfa growth was less than 30 % at 10 mg/kg of atrazine (Table 1).

2. Determination of the resistance levels of 13 alfalfa varieties *to atrazine.* The dose of the agent was determined through preliminary experiments. The results showed that at atrazine concentration of 5-160 mg/kg in soil, did not influenced FL, Crown, 9720, SD10, WL168H, ZM3, YS, SF8001, and J301 varieties growth. The inhibition rate increased with the growth of atrazine concentration,

Table 1

Afalfa varieties' sensitivity to atrazine

Varieties	Dose (mg/L)	Fresh weight inhibition rate (%)	Varieties	Dose (mg/L)	Fresh weight inhibition rate (%)	Varieties	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
Junneg2	0.20	83.24a	SF8001	0.20	5.23y	GN5	0.20	30.51r
	10.00	100.00a		10.00	61.24jk		10.00	54.11u
601	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
9720s	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	56.37s	0.20	30.19kl
	10.00	44.38st		10.00	56.37s		10.00	66.37f

and the inhibition rate of XL, YK, JU, 801, and J601 increased with the growth of atrazine concentration at 0.025-0.8 mg/kg in soil (Table 2).

The toxicity test results of atrazine to different alfalfa varieties were significantly different. And the LC_{50} difference between Juneng 2 and SF8001 was the largest. Therefore, the resistant variety was determined as SF8001 and the sensitive variety as Juneng 2 (Fig. 1).

Discussion. The advantages of chemical method of weed control are numerous: saving labor, time and ensuring good weeding effect. With the large-scale application of herbicides, some pesticide residues may impact negatively on subsequent crops. In light cases, these substances may inhibit growth and reduce yields, and in severe ones, they may cause loss and extinction of plants (Udiković-Kolić et al., 2012). Herbicide residues have become one of the main problems restricting crop rotation (Tang et al., 1998; Tappe et al., 2002; Topp et al., 2000). Atrazine is one of the broad-spectrum and efficient farmland herbicides widely used all year round. Its use has increased significantly in recent years. Unreasonable application has led to more and more atrazine residues in the soil,

Table 2

Toxicity test of atrazine to different alfalfa varieties

Varieties	Linear equation	R^2	LC_{50}	Resistance multiplier
Juneng2	y=5.3898+0.6333x	0.9958	0.2428	1.00
9720	y=2.2283+2.699x	0.7874	10.6403	43.82
ZM3	y=2.2201+2.8553x	0.8563	9.4103	38.76
J301	y=3.6013+1.41085x	0.8563	9.8409	40.53
WL168H	y=4.4206+0.7152x	0.9922	6.459	26.60
YS	y=4.1574+0.7593x	0.9426	12.873	53.02
FL	y=4.2517+0.9263x	0.9832	6.9832	28.76
HG	y=2.0161+2.896x	0.8644	10.723	44.16
XL	y=5.414+1.3251x	0.9561	0.4871	2.01
YK	y=5.3898+1.1567x	0.9387	0.460	1.89
J601	y=5.8418+1.0732x	0.9895	0.369	1.52
801	y=4.8629+1.2947x	0.9381	1.288	5.30
SF8001	y=4.0574+0.804x	0.9411	14.875	61.26

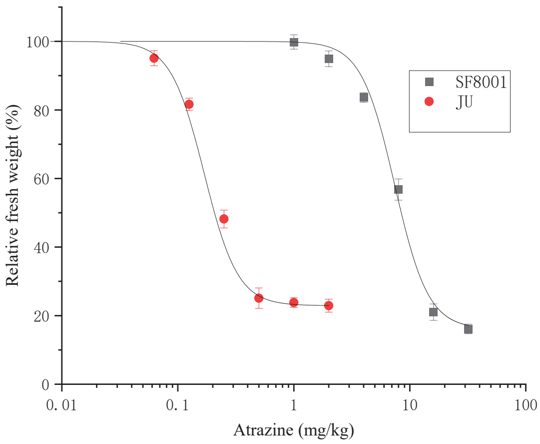

Fig. 1. Measurement curve of alfalfa sensitivity to atrazine

with the strong impact on the growth and development of other crop seeds (Solomon et al., 2008). It was found in this study that under the condition of 0.2 mg/kg atrazine, XL, YK, Juneng 2, J601, and 801 alfalfa varieties were more sensitive to atrazine. The growth and fresh weight inhibition rate of these alfalfa varieties was more than 70%, and the leaves demonstrated yellowing and wilting phenomena, while varieties as FL, HG, 9720s, SD10, ZM3, WL168HQ, YS, 8421s, SF8001, and J301 grow well under atrazine 10 mg/kg. The fresh weight inhibition rate was less than 30%, which was not significantly different from the control. The fresh weight inhibition rate was different among various alfalfa varieties, and the tolerance to atrazine significantly varied.

It was determined the changes in the activities of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase in the roots and leaves of soybeans treated with atrazine showed that the activities of these enzymes in the tolerant varieties were differ and higher than in sensitive varieties (Huang et al., 2022). Research was carried out by Griffin et al. (2013) showed that the inhibitory effect of the herbicide dicamba on soybean hypocotyl elongation is positively correlated with the dosage of dicamba. The higher treatment concentration, the more obvious the inhibitory effect of dicamba on hypocotyl elongation, but there is a difference between different soybean varieties. There were significant differences in the extent to which hypocotyls were inhibited (Griffin et al., 2013). Previous results on the tolerance test of soybeans to glyphosate and paraquat showed that there were no obvious differences in the tolerance of different soybean varieties to these herbicides (Lanie et al., 1993).

The purpose of this experiment is to screen out alfalfa varieties that are more tolerant to atrazine. Identification of high-quality tolerant germplasm resources is the basis for crop genetic improvement. Varieties with inconsistent genetic foundations have different tolerances to herbicides. Screening of alfalfa atrazine-resistant germplasm resources is of great significance to the breeding of new atrazine-resistant alfalfa varieties.

Herbicide is a chemical used to control weeds and other unwanted vegetation. Unfortunately, herbicides can cause adverse effects on ecosystems and human health (Su et al., 2018; Sukhov et al., 2014; Sun et al., 2019). Therefore, there is an urgent need to measure and monitor these effects of herbicides. IC_{50} is usually used as a judgment index for evaluating the toxicity of herbicides. IC_{50} is a measure of the chemical dose that destroy 50% of living organism. In this study, 13 alfalfa varieties suspected to be sensitive or tolerant to atrazine were screened and further tested for toxicity. The results show that the most resistant variety was SF8001 and the most sensitive variety – Juneng 2. The inhibition equation of atrazine on the above-ground fresh weight of SF8001 was y=4.0574+0.804x, the IC_{50} value was 14.875, and the resistance index – 61.26. The inhibition equation of atrazine on the above-ground fresh weight of Juneng 2 was y=5.3898+0.6333x, the IC_{50} value − 0.2428, and the IC_{50} values of the two groups were significantly different.

It was found that effect of bensulfuron residue in soil on the growth of corn seedlings, the inhibition equation of bensulfuron on above-ground fresh weight was y=0.6957x+0.694 8, R^2=0.748, IC_{10}=77.18 μg/ kg, IC_{50}=487.02 μg/kg. This is iconsstent with the method used in this experiment. Similarly, measuring the sensitivity of peanuts to bensulfuron residues showed that IC_{50} values of plant height, root length and fresh weight were 2980.95, 253.33 and 506.48 μg/kg respectively. The linear equations were y=1.2074+10916x, y=3.1017+0.7897x and y=2.4443+0.9450x, which are similar to the detection method of this test. The use of IC_{50} bioactivity assays can help detect crop sensitivity to pesticides and predict potential herbicide residue issues so growers can make better decisions about crop rotation varieties.

Conclusions. The results of this experiment show that under atrazine stress, the resistance of different alfalfa varieties to it is significantly diverse. Resistant alfalfa variety (SF8001) and sensitive variety (Juneng 2) were screened out. The IC_{50} of the SF8001 alfalfa variety is 14.875, and the IC_{50} of the Juneng 2 alfalfa variety is 0.2428, so providing a scientific basis for the breeding process of this crop in different regions and ensure a theoretical basis for the study of drug resistance mechanisms.

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Визначення стійкості люцерни до атразину

Зі стрімким розвитком тваринництва пошук ефективних і стійких кормових ресурсів став важливою проблемою для розвитку галузі. Люцерна багата на поживні речовини та відзначається швидким ростом та хорошими смаковими якостями, що привернуло увагу багатьох фермерів. Атразин є одним із гербіцидів, що широко використовується на полях кукурудзи, а сівозміна з люцерною є поширеним способом формування посівів у світі. Однак люцерна надзвичайно чутлива до гербіцидів, тому вкрай важливо вивчити стійкість люцерни до їх дії. Лабораторний біологічний аналіз – це метод визначення стійкості культур до гербіцидів, який широко застосовувався для вивчення стійкості таких культур, як люцерна та соя, до гербіциду бенсульфуронметил. Однак є кілька повідомлень про застосування методів біоаналізу для виявлення стійкості люцерни до гербіцидів. Метою цього дослідження є аналіз сирої фітомаси 60 сортів люцерни в умовах гербіцидного стресу, виявлення генотипів люцерни, стійких до скринінгу та чутливих до гербіцидів, визначення рівня стійкості сортів люцерни до гербіцидів і спроба теоретичного обґрунтування для більш глибшого розуміння механізмів стійкості до гербіцидів та покращення сортів люцерни. У цьому експерименті було використано метод токсичності ґрунту в горщиковій культурі для визначення стійкості 60 сортів люцерни. На підставі попередньо проведених тестів концентрації гербіциду розташували в такій послідовності: 5,00, 10,00, 20,00, 40,00, 80,00 і 160,00 мг/кг (для виявлення резистентності) та 0,025, 0,05, 0,10, 0,20, 0,40 і 0,80 мг/кг (для виявлення чутливості). Було визначено найстійкіший (SF8001) та найбільш чутливий сорт люцерни (Juneng 2) до атразину. IC_{50} сорту SF8001 становила 14,875, а IC_{50} сорту Juneng 2 – 0,2428. Порівняно з сортом Juneng 2, індекс резистентності (RI) SF8001 сорту становив 61,26. Результати експериментів показують, що залишки гербіцидів можуть мати токсичну дію на люцерну, а різні види люцерни мають різну стійкість до гербіцидів. Це забезпечує теоретичну базу для вивчення механізмів резистентності та наукову основу для відбору сортів люцерни для сівозміни після попередника кукурудзи.

Ключові слова: *атразин, люцерна, токсичність, стійкість до пестицидів.*