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Estimation of Pollen Viability of Metsulfuron Treated Dyers Woad *(Isatis tinctoria)* for Herbicide Efficacy Evaluation

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ABSTRACT

Viable pollen grains and eggs are necessary for processes of pollination, fertilization, and embryo formation for seed production. Various staining techniques were used to estimate pollen viability in herbicide treated plants. Effect of metsulfuron-methyl (2-[in(4-methoxy-6-methyl-l,3,5-triazine-2-yl) amino] carbonyl] amino] siilfonyl] benzoic acid) on pollen grain viability of dyers woad *(Isatis tinctoria h.)* inflorescence was investigated. Pollen grains of these plants were treated with 3,5,8,12 g a.i./ha metsulfuron in mid-anthesis stages. The treated plants were harvested in 1,3,5,7,9 and 12 day intervals after treatment and compared with control plants. Aniline blue in lactophenol (acid) and acetocarmine in glycerin (basic) were used for staining herbicide treated pollen grains. Full staining of dyers woad pollen grains significantly declined as herbicide application rates increased. Postponing the time of harvest through intervals of several days after treatment decreased the pollen grain stainability, irrespective of herbicide rate. With similar staining trends among pollen grains with the acidic and basic techniques, a significant difference in stainahility rate of the pollen grains was observed, with lower staining rates with aniline blue in lactophenol versus acetociirmine in glycerin. The rate of pollen grain stainability of herbicide treated weeds gave an adequate estimation of viability and fertility of pollen grains. As the percentage of pollen grain stainahility decreased, the efficacy of metsulfuron increased and vice versa. Use of various acidic and basic stains to estimate pollen viability can be an adequate procedure to determine the treated herbicide efficacy.

Keywords: Mcisulfuron, Staining technique , Pollen grain , Aniline blue , Acetocarmine.

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INTRODUCTION

Various staining techniques have been employed to estimate pollen grain viability and fertility. Acetocarmine (7,12,14), Alexander's stain (1,18), and aniline blue in lactophenol (5,17) are the most widely used staining techniques for pollen viability estimation. The fundamental principle in these reactions is that certain parts of plant cells are acidic and have an affinity for the basic dyes. Those molecules in the cytoplasm which have a basic character have affinities for acid dyes. Some other molecules such as protein possess both positive and negative charges, their net charge depending on the medium pH (1).

Acetocarmine in glycerin jelly acts as a basic dye with iron acting as a mordant (9).

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The nucleic acids which are strongly and negatively charged due to the presence of phosphate groups in the molecule, readily bind to basic dyes. Therefore, it is used for rapid determinations of the condition of nuclei and detailed studies of chromosomes (1,3,4,6). Helal, and Saied-Zaki (8) used acetocarmine to evaluate the effect of pollen sterility after foliar application of 2,4-D and ethephan. They found either application of 40ppm 2,4-D or 20 ppm, 2,4-D with 400ppm ethephon can act as a selective gametocide and cause complete pollen sterility. Pollen grains that did not take up stain or stained only faintly were scored as nonviahle.

Aniline blue in lactophenol stains the fertile pollen grains to dark blue without staining sterile grains (11,15). It acts as an acid dye and becomes bound to cytoplasm but does not stain nuclei clearly (13). Springer *et at* (17) used aniline blue in lactophenol stain to estimate pollen viability in big bluestem (Andropogon gerardii Vitman) as related to spikelet type. They found an average pollen viability in sessile spikelcts that was 2.3 times those in pedicellate spikclets. They suggested that heritable variation may magnify the pollen variability among various spikelets.

Dyers woad is a troublesome weed of the mustard family that is rapidly spreading onto crop and range lands of temperate zones of Asian countries, North Africa, Europe, Australia and several states of the United States (2,19). It is totally dependent upon seed production and seed movement for its dissemination into uninfested territories. Lack of viable pollen grains and/or eggs will inhibit its seed production. Melsulfuron application to dyers woad in mid-anthesis inhibits seed production of treated plants (10).

The objective of this study was to investigate whether staining techniques can be used for estimating pollen viability of metsulfuron treated dyers woad and thereby determining the herbicide efficacy.

MATERIALS AND METHODS

The 125 day dyers woad vernalized seedling rosettes were arranged in a completely rand-omized design in the greenhouse. The plants were treated with metsulfuron when bolted and their inflorescence were in the mid-blooming stages. Treatments included 0,3,5,8 and 12 g/ha metsulfuron and were replicated lour times by using 4 pots, each with 4 rosettes. Herbicide was applied using an enclosed spray chamber that delivered 187 L/ha of water at 207 KPa. The sprayer was equipped with a single flat fan 8001 Teejet nozzle. On 1,3,5,7,9 and 12 days after herbicide trea-tment, a random sample cluster of racemes from each pot was harvested and fixed in Carnoy's (6:3:1 v/v of 95% ethanol: chloroform: acetic acid) solution. The fixing solution in each sample vial was replaced after 24 h with a 70% ethyl alcohol (EtOH) and stored in a refrigerator until analyzed.

To prepare acetocarmine in glycerin jelly a quarter gram of finely powdered carmine was added to 50 ml of freshly melted glycerin jelly. To dilute, 50 ml distilled water was added to it and mixed. The aniline blue in lactophenol was prepared according to Radford *et al.* (19) by mixing 20 ml of melted dry phenol with 40 ml glycerin and 40 ml of distilled water. To this solution, one to five drops of 1% aqueous solution of aniline blue were added. Aniline blue in lactophenol, like acetocarmine in glycerin, is able to stain viable pollen grains.

To determine the adequate floral bud stage of dyers woad the racemes of the control plants were harvested and fixed in Carnoy's solution. The developmental stages of buds were determined by measuring the size and length of buds, length of anthers and size of pollen grains (table 1). Anthers from three buds in each stage were randomly selecied and their pollen grains stained with aniline blue in lactophenol or acetocarmine in glycerin.

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The results of this test were used as basis for selection of adequate floral bud stage of development for herbicide treated plants.

Dyers woad inflorescences were harvested from the greenhouse and evaluated in the laboratory for pollen grain stainability. To select the latest stage of floral buds and their pollen grains, the largest buds of racemes on each sample were determined. From among these buds, five were randomly chosen and their anthers smashed on a microscopic slide. Empty anther shells were removed and one drop of the respective staining solution was applied to the pollen grains on the respective slide. After uniformly dispersing the pollen and stains they were covered with a cover slip. Thirty minutes later the slide was observed in a Olympus BH-2 microscope with a 20X objective and 10X ocular lens.

In both staining techniques, the smeared pollen grains were categorized into three staining groups, fully stained, semi, and non-stained pollen grains. Two hundred pollen grains were counted for replication of each treatment.

To evaluate the fully stained pollen grains, a factorial design with time of harvesting (six levels: 1,3,5,7,9 and 12 day after metsulfuron application), rate of application (six levels: 0,3,5,8 and 12 g a.i./ha metsulfuron) and staining technique (comparing aniline blue and acetocarmine) was formed. Natural dispersal of pollen grains in control plants ended within 7 days after application. The average nontreated natural dispersal of pollen grain was considered as 9 and 12 days after application of control plants.

RESULTS AND DISCUSSION

Various techniques were tested to estimate the pollen viability of metsulfuron treated dyers woad inflorescence fixed in Carnoy's solution. Germination test and 2.3.5-triphenyl tetrazolium (TTC) were not feasible for determining viability of prc-fix pollen grains. Aniline blue in lactophenol and acetocarmine in glycerin jelly were found to be the best techniques for estimating dyers woad pollen grain viability using fixed anthers. These solutions were extremely active in staining the viable pollen grains and showed sharp differences in smearing advanced pollen grains from abnormal ones. Acetocarmine in glycerin jelly and aniline blue in lactophenol gave sharp red and blue color to viable pollen grains, respectively. The undeveloped or herbicide-suppressed pollen grain showed light staining or partial staining or no staining with any staining technique. As a result, only sharply stained pollen grains were considered as potential viable ones. At least 15 minutes were required for each staining solution to sharply stain the non-herbicide-treated pollen grains. None of the non-stained or partially stained pollen grains became more intensely stained by being left in the staining solution for a longer time. Therefore, pollen grains were evaluated 30 minutes after staining.

From floral bud initiation to fruit maturation of dyers woad, eight easily distinguishable stages including three pre-blooming, blooming and four post blooming stages were determined (Table 1). None of the pollen grains were stained by any staining technique in stages one and two, whereas almost all of the pollen grains in stages three and four were clearly stained with either aniline blue or acetocarmine. Fresh pollen grains also gave similar results when compared with fixed pollens in these stages. These observations indicated that only pollen grains in stage three or four had the ability to fertilize embryos. Therefore, buds in these stages from each treatment in the pollen viability experiment were used for running pollen grain viability tests of metsulfuron-treated plants.

Development of herbicide-treated buds was inversely related to the rate of herbicide. Some of the buds, in late pre-blooming stages,

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Table 1. Identification key for dyers woad inflorescence developmental stages

Stage	Key characteristics
51	Floral buds initiation as compact juvenile clusters, hidden by dark green or black bracts. Bud size <0.5 mm diameter.
52	Floral buds visible as clusters open revealing bright / yellow green buds. Advanced buds size 0.5 to 1 mm diameter.
53	Raceme separation complete, advanced pedicellate oval shaped buds with visible yellowish sepals and petals. Advanced buds size > 1 mm diameter, 2 mm long.
54	Blooming-Anthesis, advanced basal flowers with extended sepals and petals dehiscent anthers and pollination completed.
55	Early fruit formation, silicles with dried sepals attached. Silicle 2 mm diameter, 5 mm length.
56	Seed dough stage, milky seeds 0.5 diameter, 2 mm long. Silicle 3 mm diameter, 10 mm length.
57	Fruit hardening, Silicles and seeds at final size and shape, and solidified. Seed 1.3 mm diameter, 3 mm long. Silicle 6 mm diameter, 15 mm long.
58	Fruit maturation, fruit dark purplish-brown or black, plants dried/dead, seeds matured with light brown color and oblong shape.

were observed to blossom when treated with 3 g/ha herbicide. None blossomed when treated with 5 g/ha or higher. The number of flowering buds on low dosage herbicide treated plants reduced. These buds were needed for pollen grain analysis. More racemes of inflorescences were used to get enough adequate size pollen grains.

The stainability of metsulfuron-treated pollen grains to acetocarmine and aniline blue is listed in Table 2. Partially stained, nonstained or weakly stained pollen grains were considered nonviable and nonfertile. Stainability and size of pollen grains were noted and classified as either fully stained, light or partially stained and nonstained pollen grains. Examination of the 200 pollen grains showed a highly significant difference atlributed to harvest day, herbicide rate and staining techniques.

The viability of pollen grains decreased with time after herbicide application (Fig. 1), due to an increase in level of metsulfuron in reproductive tissues. There was an indirect relationship between the level of treated herbicide and amount of branched chain amino acids (16). More developed pollen grains could require fewer nutrient and amino acids, or could absorb from free and nonbound amino acids in the phloem. Full staining of dyers woad pollen grains declined as more herbicide was applied (Fig. 2). Viability of pollen grains from herbicide treated plants declined sharply compared to the control (untreated) plants.

Herbicide action prevented the stainability of pollen grains either non-stained, or semi- and partially stained. The developmental stage of individual buds at the herbicide application time and rate of metsulfuron application determine the extent of advancement of each bud after herbicide application. The relationship between semi-or nonstained pollen grains and rate of herbicide treatment was not meaningful using either staining technique (Fig. 3 and Fig. 4). Significant reductions in number of fully stained pollen grains, treated

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Table 2. Melsulfuron-treated dyers woad pollen grains stained with acetocarmine in glycerol and aniline blue in lactophenol.

Harvest	metsulfuron	Acetocarmine			Aniline blue		
(DAT)[a]	g/ha	full[b]	semi[c]	non[d]	full	semi	non
1	1)	L90	6	4	176	17	7
	3	179	19	2	166	14	20
	5	134	30	36	147	12	41
	8	164	16	20	142	51	7
	12	99	14	87	111	52	37
3	0	188	2	10	188	7	5
	3	167	15	18	127	62	11
	S	154	4	42	112	53	35
		140	7	53	106	48	46
	12	72	15	113	102	30	68
5	0	194	2	4	190	8	2
	3	107	28	65	90	70	40
	5	144	20	36	89	72	39
	8	68	10	122	63	40	97
	12	107	45	48	52	76	72
7	0	194	4	2	162	33	5
	3	119	40	41	UK)	75	25
	5	104	27	69	SO	46	65
	8	89	55	56	50	96	54
	12	25	80	95	20	113	67
9	0	192	3	5	179	16	5
	3	45	43	112	55	83	62
	5	68	42	90	63	59	78
	8	32	96	72	51	65	84
	12	71	37	92	49	62	89
12	0	192	3	5	179	16	5
	3	35	66	99	24	31	145
	5	3	26	171	14	35	151
	8	16	98	86	11	39	150
	12	15	45	140	2	16	182

a Days after treating dyers woad panicle with various rates of metsulfuron. b Full stained, c Semi-stained, d Non-stained

with metsulfuron, was due to the adverse effect of the herbicide on pollen grain development.

There was a significant interaction between herbicide rate and harvest interval after application. The highest percentage of fully stained pollen grains occurred at the lowest metsulfuron rate and vice versa. Even though there were similar staining trends among pollen grains with the acidic and

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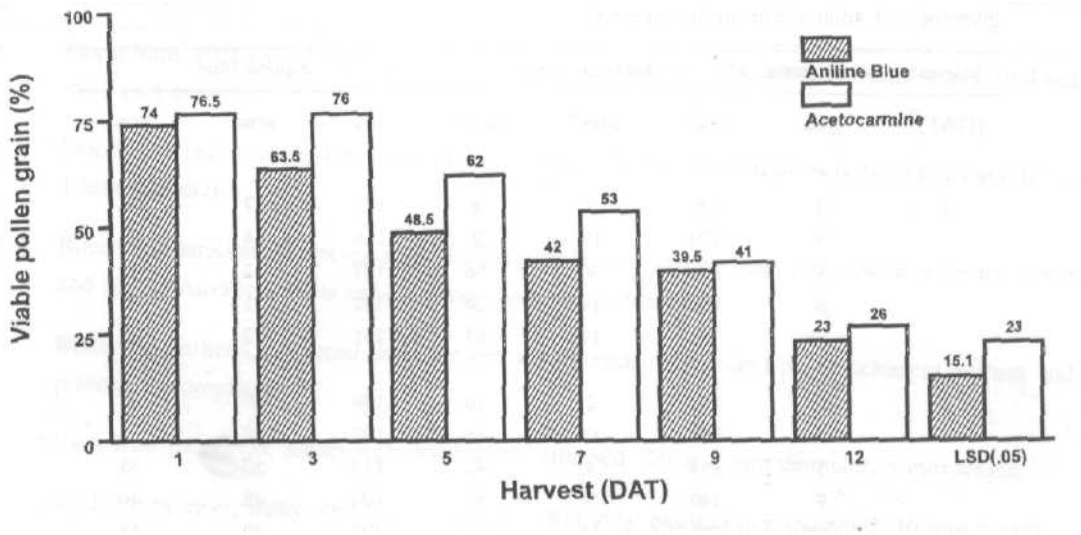

Figure l. Percent viable pollen grains of metsulfuron-treated dyers woad harvested at various of day intervals after treatment (DAT). Data are averaged over herbicide rate and techniques.

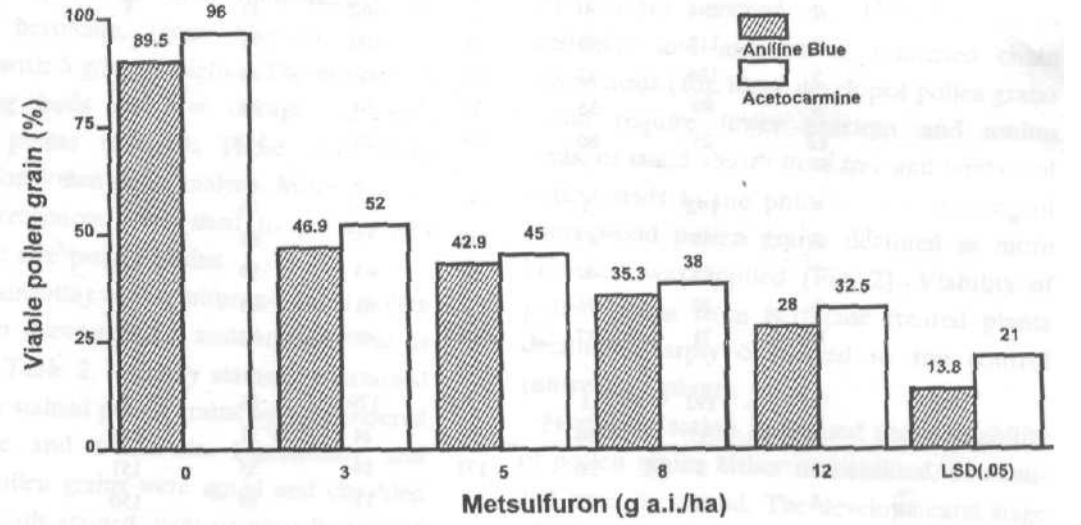

Figure 2. Percent viable pollen grains of dyers woad treated with various rates of metsulfuron in preanthesis stages. Data are averaged over DAT, and staining techniques.

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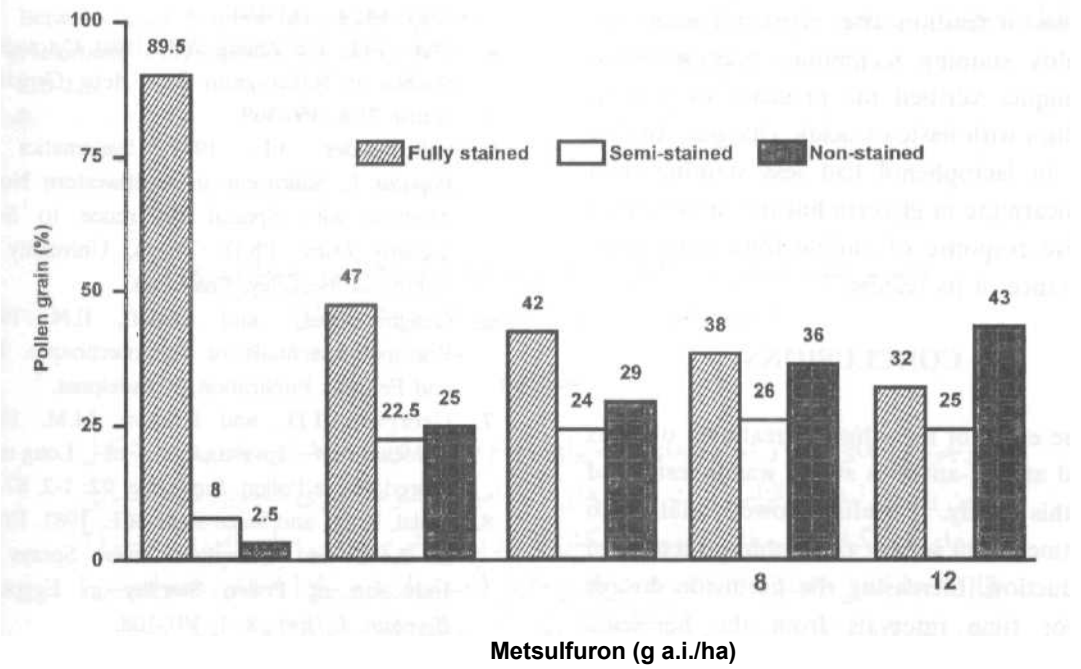

Figure 3. Percent full stained, semi-and nonstained pollen grains of metsulfuron-treated dyers woad using aniline blue in laclophenol. (Data are averaged over DAT).

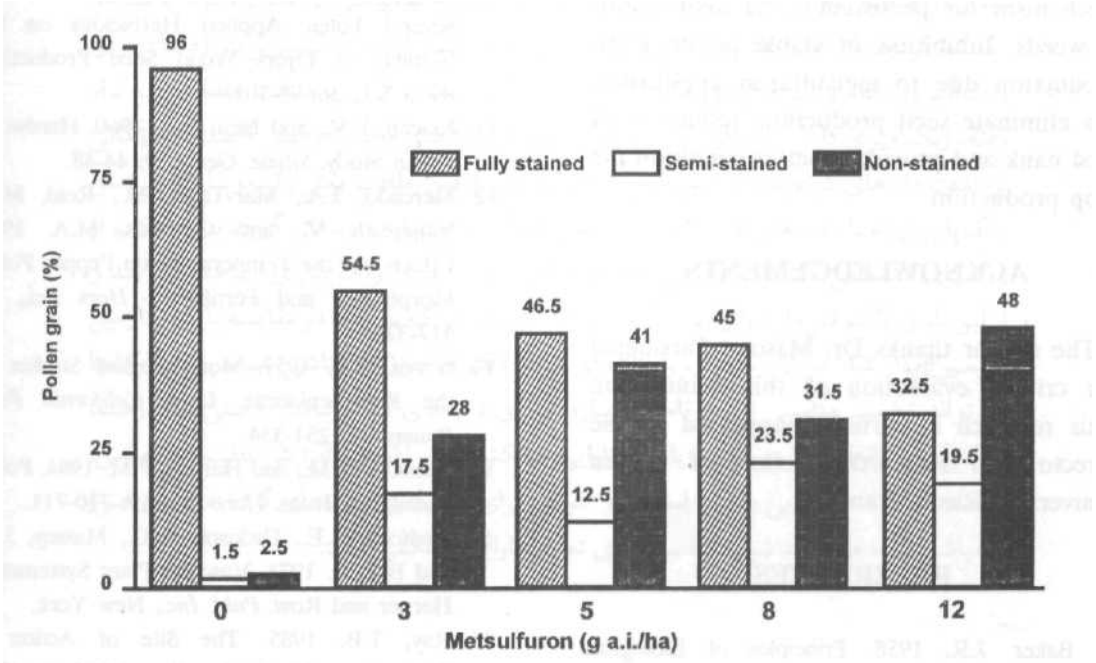

Figure 4. Percent full stained, semi-and nonstained pollen grains of metsulfuron-treated dyers woad using acetocarmine in glycerin. (Data are averaged over DAT).

basic techniques, but the difference was significnat in stainability of pollen grains. Aniline blue in lactophenol gave a lower staining percentage compared to acetocarmine

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in glycerin. Lack of fresh pollen grains in the process of fixation after harvest limited the viability staining techniques because these techniques verified the presence of protein residues with basic or acidic charges. Aniline blue in lactophenol had less staining than acetocarmine in glycerin but the more conservative response of aniline blue gives more assurance of its results.

CONCLUSIONS

The effect of metsulfuron treatment of dyers woad at mid-anihesis stages was investigated in this study. Results showed that such treatment can inhibit the viable pollen grain production. Increasing the herbicide dosage and/or lime intervals from the herbicide treatment to pollen harvesting, lowered the number of viable pollen grains (table 2). Seed production is a very important survival mechanism for persistence and distribution of weeds. Inhibition of viable pollen grain production due to metsulfuron application can eliminate seed production, reduce weed seed bank and provide a better condition for crop production.

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تخمین قوه حیاتی دانه‌های گرده علف هرز نیل وحشی سمپاشی شده با متسولفورون برای ارزیابی کارایی علفکش

چکیده

وجود دانه‌های گرده و تخمک‌های زنده برای انجام فرایندهای گرده‌افشانی، باروری و تشکیل جنین برای تولید بذر الزامی است. روش‌های رنگ‌آمیزی متنوعی برای تخمین قوه حیاتی دانه‌های گرده گیاهان سمپاشی شده با علفکش به کار رفت. اثر سمپاشی متاسولفورون متیل (۲ [[[[(۴-متوکسی-۶متیل-۱ ، ۳ ، ۵-تریازین-۲یل (آمینو]کاربونیل] آمینو] سولفونیل] بنزوییک اسید) بر روی قوه حیاتی دانه‌های گرده گل آذین علف هرز نیل وحشی (*Isatis tinctoria* L.) مورد بررسی قرار گرفت. در اواسط به گل نشستن این گیاهان با ۳ ، ۵ ، ۸ و ۱۲ گرم ماده مؤثر در هکتار علفکش متاسولفورون متیل سمپاشی شدند و دانه‌های گرده هر تیمار در فواصل زمانی ۱ ، ۳ ، ۵ ، ۷ ، ۹ و ۱۲ روز بعد از سمپاشی برداشت شد و با شاهد مقایسه گردید. از انیلین بلوی لاکتوفنل‌دار (اسید) و استوکارمین گلیسیرین‌دار (قلیا) برای رنگ‌آمیزی دانه‌های گرده گیاهان سمپاشی شده استفاده شد. با افزایش غلظت علفکش به‌کار رفته، رنگ‌آمیزی کامل دانه‌های گرده به طور معنی‌داری کاهش یافت. افزایش فاصله‌های زمانی برداشت دانه‌های گرده بعد از سمپاشی، بدون در نظر گرفتن غلظت بکار رفته، باعث کاهش رنگ‌پذیری تعداد دانه‌های گرده شد. علیرغم رنگ‌پذیری مشابه دانه‌های گرده با استفاده از روشهای شیمیایی اسیدی و قلیایی، تفاوت معنی‌داری در میزان رنگ‌پذیری آنها مشاهده شد و انیلین بلوی لاکتوفنل‌دار دارای مقدار کمتری دانه‌های گرده رنگ پذیرفته در مقایسه با استوکارمین گلیسیرین‌دار شد. میزان رنگ‌پذیری دانه‌های گرده علفهای هرز سمپاشی شده با علفکشها می‌تواند تخمین مناسبی از قوه نامیه و بارورسازی دانه‌های گرده باشد. وقتی که درصد رنگ‌پذیری دانه‌های گرده کاهش یافت، کارایی علفکش متسولفورون افزایش یافت و بالعکس. استفاده از مواد شیمیایی رنگزن اسیدی و قلیایی برای رنگ‌آمیزی دانه‌های گرده علفهای هرز سمپاشی شده می‌تواند شیوه مناسبی برای تعیین کارایی علفکش باشد.

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