Acta Physiol Plant (2007) 29:115–120
DOI 10.1007/s11738-006-0014-1

ORIGINAL PAPER

The effects of Fusilade (Fluazifop-*p*-butyl) on germination, mitotic frequency and α-amylase activity of lentil (*Lens culinaris* Medik.) seeds

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Received: 17 January 2006 / Accepted: 31 May 2006 / Published online: 23 February 2007

Abstract In this study, seed germination percentages, effects on phases of mitosis and α-amylase enzyme activity of lentil seeds treated with four different concentrations (0.25, 0.5, 1 and 1.5%) of Fusilade (Fluazifop-*p*-butyl) were determined. Median EC (effective concentration) values were calculated according to seed germination percentages after treatment for 72 h. Germination percentages of primary lentil roots decreased with increasing Fusilade concentrations. Cytological observations showed that the mitotic frequency in root meristematic cells were decreased parallel to the increase in concentrations and all Fusilade concentrations applied decreased the activity of α-amylase enzyme in lentil seeds. The obtained results indicate that the herbicide Fusilade had the ability to cause reduction in seed germination, mitotic frequency and also α-amylase activity of lentil seeds.

Keywords α-Amylase · Fluazifop-*p*-butyl · Lentil (*Lens culinaris* Medik.) · Mitosis · Seed germination

Communicated by W. Bielawski.

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Introduction

Different pesticides and plant growth regulators are being used extensively in modern agriculture; though the use of these chemicals has become a necessity, their frequent and indiscriminate use has many undesirable consequences in culture plants.

Fusilade (Fluazifop-*p*-butyl) is a post-emergence phenoxy herbicide which is used in lentil fields for weed control. It is absorbed rapidly through leaf surfaces and quickly hydrolyzes to fluazifop acid. The acid is transported primarily in the phloem and accumulates in the meristems where it disrupts the synthesis of lipids in susceptible species (Urano 1982; Erlingson 1988). Fusilade can pass readily into fish tissue and is highly toxic to fish and other aquatic species, including invertebrates (Daphnia 48 h LC50 > 10 mg/l) (EXTOXNET 1996). It has been shown to inhibit fungal growth (Abdel-Mallek et al. 1996; Gorlach-Lira et al. 1997). But there is limited information about the phytotoxic effects of Fusilade on culture plants (EXTOXNET 1996).

Lentil (*Lens culinaris* Medik.) is a major grain legume crop in many developing countries in West Asia, North Africa and many other areas of the world. (Turk et al. 2003). Lentil is not very competitive (especially as seedlings) with many of the grasses and/or broadleaf weed species that infest farm fields, so weed control before planting and early in the growing season is critical.

Fayez and Kristen (1996) hypothesized that residues of some herbicide compounds would effect crop seedlings during very early stages of their development and they stated that the radicule is the first organ to come directly into contact with herbicide in the soil. Boutin

et al. (2000) reported that *Sinapis arvensis* L. and *Phaseolus vulgaris* L. exhibited marked effects on the vegetative growth and reproductive performance when sprayed at 10% label rate with metsulfuron methyl and the seedling stage was the most sensitive period for all species tested. The thiocarbamate herbicide, triallate caused root growth retardation 6 months after application to the soil (Beestman and Deming 1976).

According to our knowledge, seed germination and mitosis have not yet been examined in relation to the action of the phenoxy herbicide Fusilade. The present investigation was conducted to understand the root growth inhibiting effect of Fusilade on lentil seeds. The objectives of this investigation were: to determine the effect of different concentrations of Fusilade on mitotic frequency of seed germination percentages, phases of mitosis and α-amylase enzyme activity of lentil seeds.

Materials and methods

Treatment of lentil seeds with Fusilade

Seeds of *Lens culinaris* Medik. cv. Sultan were pretreated with 5% NaOCl for 10 min for seed surface sterilization, then placed under clean bench conditions in Petri dishes and filled with boiled tap water at room temperature for soaking and germination. At the beginning of the study, six different Fusilade concentrations 0.125, 0.25, 0.5, 1, 2 and 4% were prepared in modified Hoagland nutrient solution (Ouzounido et al. 1997). The control groups were treated only with modified Hoagland nutrient solution. The EC50 (the concentration at which germination was 50% of the control) values were calculated according to seed germination percentages with ''Trimmed Spearman Karber'' method after treatment for 72 h (Bartakova et al. 2001; An 2004). The root tips which were 0.5 mm and higher were determined as germinated. All experiments were done in three repetitions. The germination of seeds was observed in laboratory conditions in petri dishes for 5 days.

Mitotic frequency analysis

The root tips were fixed in Carnoy solution (3:1, alcohol: asetic acid) and hydrolysed in 1 N HCl at 60°C for 5–10 min followed by squashing in a 2% orcein stain in 45% acetic acid. Slides were kept in a freezer and examined in a month (Rank and Nielsen 1994). The mitotic frequency was determined by examination of 500 cells per slide and calculated as mitotic cells per approximately 2,000 cells. Three replicates were made for each concentration.

The extraction of α-amylase enzyme

Lentil seeds were surface strelized in 15% NaOCl for 1 min then washed for 10 min, three times. Sterilized seeds were germinated at 25°C for 48 h. The radicules of germinated seeds were cut out. Seeds were homogenized with 50 mM Malate buffer solution (pH: 5.2, 50 mM NaCl, 2 mM $CaCl_2$) for 5 min and stored at room temperature for 30 min. Then santrifuged at 5,000 rpm for 8 min and the supernatant was taken as enzyme source.

Measurement of α-amylase activity

α-Amylase activity was measured using dextrogenic method. This method is based on the reaction between starch and iodine. When starch is treated with iodine, purple color is observed, however this color gets lighter as starch breaks down. During activity measurements, extracts obtained from non-treated seeds and treated seeds with different Fusilade concentrations were used as the enzyme sources, and 1% soluble starch as the substrate. Activity measurements were realized by observation of the lightening of the color of the enzyme-substrate mixture using a spectrophotometer. The α-amylase activity was measured by calculation of the amount of substrate (starch) loss. For this purpose, blind, control and sample tubes were prepared (Ekinci and Aktac 1997).

(a) Preparation of the blind tube: 9 50 mM Malate buffer (pH = 5.2), 0.1 enzyme extract, 0.9 ml 1 N HCl.
(b) Preparation of the control tube: 7 ml 50 mM Malate buffer (pH = 5.2), 2 ml 1% starch, 0.1 ml enzyme extract, 0.9 ml 1 N HCl.
(c) Preparation of the sample tube: The content is the same as the control tube, however, 0.9 ml 1 N HCL solution was added following the incubation period (4 min).

Blind, control and sample tubes that were prepared as described above were incubated in water bath for 4 min at 30°C. At the end of this period, the reaction in the sample tubes were stopped by addition of 0.9 ml 1 N HCl. To give color to the starch, 1 ml iodine solution (0.3% I, 3% KI) was added to all of the tubes. The starch amount left following the enzymatic breakdown was read from the sample tube and compared to the blind and control tubes using a spectrophotometer set at 620 nm wavelength (Jenway 6105 UV/VIS Spectrophotometer). The net starch amount broken by the enzyme is calculated by

subtraction of the absorbance amount in the sample tubes from the absorbance amount in the control tubes. α-Amylase activity was calculated in units/ml from the starch standard curve prepared before (Ekinci and Aktac 1997). One unit of α-amylase activity was defined as the enzyme amount that transforms 1% starch amount into the product at 30°C (pH = 5.2) in 4 min time.

Results

The effect of Fusilade concentrations on seed germination percentage

After the treatment with different concentrations of Fusilade, we observed that the percentages of seed germination were decreased while the concentrations were increased (Table 1). EC50 values were determined between the concentrations 1 and 1.5% (On the third day, the difference between the concentrations can be seen easily). Germination of *Lens culinaris* seeds on the fifth day is seen in Fig. 1.

The effects of Fusilade concentrations on mitotic frequency

As seen in Table 2, all the Fusilade concentrations used had a negative effect on mitotic division (Table 2). The mitotic frequency was reduced with the increase in concentrations (Fig. 2). The mitotic frequency was 13.93% in the control group, and decreased to 8.9 in 0.25% concentration and 6.69 in 0.5% concentration, but in 1% concentration it was increased to 6.96%. During our observations of seed germination for 5 days, there was also an increase in root growth in 1% concentration. In 1.5% concentration, mitotic frequency was reduced as 1/3 of control and determined as 4.87%.

The effect of Fusilade concentrations on different phases of mitosis

Different concentrations of Fusilade reduced the number of phases of mitosis when compared with control (Fig. 3). Especially when we compare the concentrations 1 and 1.5%, there was a half reduction in all phases of mitosis in 1.5% Fusilade concentration.

Table 1 The effect of Fusilade concentrations on seed germination percentage

Fusilade concentration (%) (v/v)	Seed germination percentage (%) ± SD				
	1stday	2nd day	3rd day	4thday	5thday
Control	46 ± 0.23	76 ± 0.14	92 ± 0.33	92 ± 0.20	92 ± 0.08
0.125%	40 ± 0.35	92 ± 0.33	96 ± 0.11	96 ± 0.13	96 ± 0.19
0.25%	32 ± 0.22	72 ± 0.25	84 ± 0.16	86 ± 0.18	86 ± 0.26
0.5%	12 ± 0.10	46 ± 0.30	62 ± 0.20	72 ± 0.27	72 ± 0.15
1%	4 ± 0.34	44 ± 0.12	56 ± 0.23	64 ± 0.06	82 ± 0.26
2%	–	2 ± 0.01	8 ± 0.09	8 ± 0.21	8 ± 0.11
4%	–	–	4 ± 0.22	4 ± 0.14	4 ± 0.19

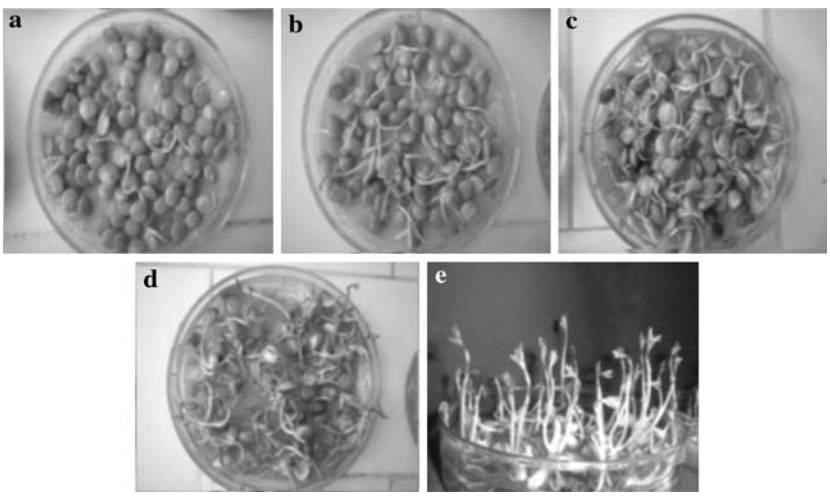

Fig. 1 Germination of *Lens culinaris* seeds in 5th day. **a** 1.5% Fusilade, **b** 1% Fusilade, **c** 0.5% Fusilade, **d** 0.25% Fusilade, **e** control

Table 2 The effect of Fusilade concentrations on mitotic frequency percentage

Fusilade concentration % (v/v)	The number of total counted cells	The number of divided cells	Mitotic frequency (%)
Control (%)	2,160	301	13.93
0.25	2,100	188	8.90
0.5	2,120	142	6.69
1	2,010	140	6.96
1.5	1,600	78	4.87

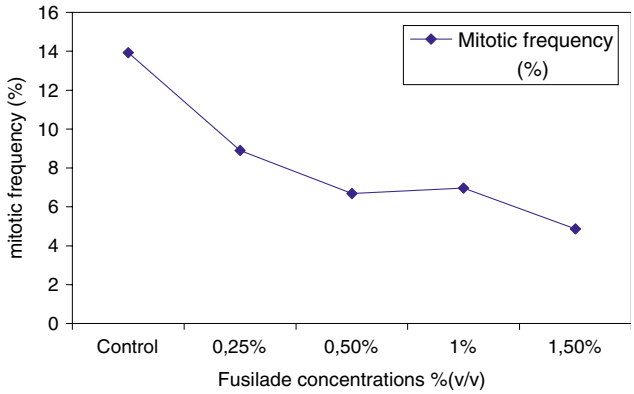

Fig. 2. The effect of Fusilade concentrations on mitotic frequency percentage

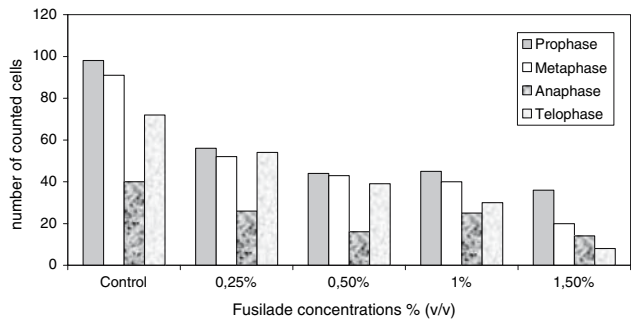

Fig. 3 The effect of Fusilade concentrations on different phases of mitosis

The effect of fusilade concentrations on α-amylase activity of lentil seeds

All the used concentrations caused a decrease in α-amylase activity and it was observed that the concentrations 0.25, 0.5 and 1% made a half-reduction of enzyme activity when compared with the control. But there was no significant difference between the concentrations. Also, the last concentration of 1.5% made a reduction of ¼ when compared with the control (Table 3). The remaining activity and inhibition (%) was 24.19 and 75.81, respectively in 1.5% Fusilade concentration.

Table 3 The effect of Fusilade concentrations on α-amylase activity of lentil seeds

Fusilade concentration (%) (v/v)	Enzyme activity (Uml^{-1})	Remaining activity (%)	Inhibition (%)
Control (%)	1.24	100	–
0.25	0.50	40.30	59.7
0.5	0.50	40.30	59.7
1	0.50	40.30	59.7
1.5	0.30	24.19	75.81

Discussion

Disturbance of one of seed germination and mitosis could produce severe consequences for root growth and development. This was clearly demonstrated for the roots of *Pisum sativum* and *Zea mays* with different concentrations of the sulfonylurea herbicides chlorsulfuron and metsulfuron methyl which caused severe injuries of the root growth (Fayez et al. 1994).

Ruiz-Santaella et al. (2003) found that the herbicide cyhalofop-butyl from the family of phenoxy acetic acid decreases seed germination by 50% in *Echinochloa muricata*. Sasaki et al. (1968) reported that the use of herbicide 2,4–dicloro-phenoxy acetic acid (2,4-D) in *Pinus*, decreased the seed germination percentage and similar results were found by Chopra and Singh (1978) in *Guizotia* which was treated with the same herbicide 2,4-D. Also the studies with different pesticides showed that seed germination percentages decreases parallel to the increse in concentrations. In *Lens culinaris* (Aktaç et al. 1994) the use of insecticide endosulphan and in *Allium cepa* (Dane and Dalgıç 2005) the use of fungicide benomyl decreased seed germination percentages with increasing concentrations.

In this investigation it was observed that the use of higher concentrations of Fusilade decreased the number of phases of mitosis. It was also seen that the frequency of prophase was higher in all Fusilade concentrations as well as the control (Dalgic 2005). In other studies with the herbicides, similar results were obtained, metribuzin in *Vicia faba* (Soliman and Ghoneam 2004) and tribunil in *Allium cepa* (El-Khodary et al. 1990) made an increase in prophase cells in root meristematic cells. Plants treated with herbicides that cause disruption of cell division have mitotic stages present, but sometimes one or more stages normally present will be absent or aberrant. Inhibition of cell division is a secondary effect caused by some

disturbance of a plant's metabolic process (Hess 1983; Kim and Bendixen 1987).

The symptoms induced by fluazifop-butyl in *Acanthospermum hispidum* were wilting and necrosis. In *Avena sativa*, lipid biosynthesis and acetyl-CoA carboxylase (ACCase) activity were inhibited, and electrolyte leakage from the shoots was increased by fluazifop-butyl (Xiao and Hiroshi 2002).

The cross-resistance to fenoxaprop-*P*-ethyl, fluazifop-*P*-butyl, propaquizafop, in *Lolium* resistant to diclofop-methyl were evaluated in a study. The parameters, germination (%), number of roots or root length did not show a good relation between the concentration and its efficacy (curves of concentration response) for any of the susceptible and resistant biotypes studied (Michitte et al. 2003).

The enzyme α-amylase is capable of hydrolyzing α-1,4 (glucose–glucose) linkages in the middle of the starch molecules. The activity of α-amylase which breaks starch down and gains energy affects plant growth. According to our findings, α-amylase activity decreases with increase in Fusilade concentrations and during the same period mitotic frequency decreases, so growth was inhibited (Dalgic 2005). The result of this study showed that α-amylase activity is more sensitive to herbicide Fusilade than seed germination in lentil against to herbicide Fusilade. Lentil plant isn't tolerant to higher concentrations of Fusilade.

The Fusilade treatment in root meristem cells of *Lens culinaris* with four different concentrations resulted in reduction in germination of seeds, mitotic frequency of root tip cells and α-amylase activity of lentil seeds. As we know, mitotic frequency reflects cell division frequency and is used to determine the root growth ratio as a significant parameter. Regardless of which process is affected, both processes result in a reduced supply of new cells in the root meristem leading to an eventual inhibition of growth.

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