# Safeners as Corn Seedling Protectants against Acetolactate Synthase Inhibitors*

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*Zea mays* L., *cv.* Potro, shoots had a higher level of resistance to imazaquin (IQ) and metsulfuron methyl (MSM) than roots. Shoot lengths were increased by pretreating the seeds with commercial 1,8-naphthalic anhydride (NA) at 1% (w/w) or oxabetrinil at 0.2% (w/w). The growth of shoots of safened seeds was unaffected by 400 nM IQ and by 40 nM MSM.

The *in vivo* activity of acetolactate synthase (ALS) extracted from corn shoots and roots was not affected by treatments with IQ or MSM, but pretreatment of seeds with NA or oxabetrinil, prior to germination, caused an increase in the level of extractable ALS from shoots. ALS activity from roots and shoots of NA-pretreated seedlings was increased to a large degree (>40%) when the seedlings were germinated on 40 nM MSM, whereas ALS activities from oxabetrinil-pretreated seedlings were enhanced to a lesser degree (≈20%). ALS from unsafened seedlings was inhibited 21% by 400 nM IQ and 70% by 40 nM MSM *in vitro,* but ALS from roots of seedlings germinated on 400 nM IQ was not inhibited by 400 nM IQ *in vitro.*

## Introduction

Imidazolinones and sulfonylureas are potent herbicides which kill plants by interfering with the biosynthesis of the branched chain amino acids [1–5]. These herbicides are slow, tight-binding inhibitors of plant acetolactate synthase (ALS) *in vitro* [6, 7]. The objective of this study was to determine the sensitivity of a corn line to imazaquin (IQ) and metsulfuron methyl (MSM) at the whole seedling level as well as at the extractable ALS level when the seedlings used were safened or not by dressing the seeds with 1,8-naphthalic anhydride (NA) or oxabetrinil safeners before treatment with IQ or MSM.

## Materials and Methods

### *Chemicals*

IQ was extracted from commercial growth regulator "Cycocel® CL" and technical grade MSM (99.2% purity) was a gift from Du Pont Company. They were dissolved in buffer (50 mM phosphate, pH 7.0) (400 µmol/l), and further diluted in 5 mM citrate-phosphate buffer (pH 5.0) to the desired concentration. NA was from Aldrich and oxabetrinil was a gift from Ciba-Geigy Limited.

### *Plant material*

Corn (*Zea mays* L. *cv.* Potro) seeds were supplied by Maïsadour (Mont de Marsan, France). They were sown on one filter paper in square dishes and moistened with 60 ml of herbicide solution in buffer (5 mM citrate-phosphate, pH 5.0). The used herbicide concentrations were 0, 40, 100, 400 and 1000 nM for IQ and 0, 4, 12 and 40 nM for MSM. The seedlings were grown in darkness for 3 d with a constant temperature of 28 °C.

### *Root elongation study*

After 3 d, roots and shoots were harvested for length and fresh weight determinations. Results from length and fresh weight measurements were similar so only the length data, expressed as the net growth, are presented. They are expressed as the mean of three replicates.

### *ALS extraction and determination*

The method used was similar to described procedures [2, 3, 7–10] with modification in the preparation of plant extracts and in the enzyme assays.

Plant material was ground with a small amount of Fontainebleau's sand in six volumes (fresh-weight basis) of cold 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM sodium pyruvate,

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0.5 mM magnesium chloride, 0.5 mM thiamine pyrophosphate chloride (TPP), 10 μM flavin adenine dinucleotide (FAD), 1 mM dithiothreitol and 1% (w/v) polyclar AT, using a prechilled mortar and pestle. The homogenate was clarified by centrifugation at 27,000 × *g* for 15 min at 4 °C. The supernatant fraction was brought to 50% (v/v) saturation with saturated cold ammonium sulfate solution and allowed to stand for 1 h on ice. Then ALS was pelleted by centrifugation at 20,000 × *g* for 15 min at 4 °C and the supernatant was discarded. The precipitated pellet was resuspended in cold 100 mM potassium phosphate buffer (pH 7.5) containing 20 mM sodium pyruvate and 0.5 mM magnesium chloride. The dissolved proteins ('enzyme solution') were placed on ice and used directly for ALS and protein determinations.

ALS activity was assayed by mixing the enzyme solution with a reaction buffer. The reaction mixture in 0.5 ml total volume contained 40 mM sodium pyruvate. 0.5 mM magnesium chloride, 0.5 mM TPP, 50 μM FAD, various concentrations of IQ, MSM, NA or oxabetrinil and 0.2 ml enzyme solution, in 40 mM potassium phosphate buffer (pH 7.0). IQ and MSM was dissolved in buffer (50 mM phosphate, pH 7.0) (400 μmol/l) and diluted as appropriate with 20 mM potassium phosphate buffer (pH 7.0). Incubations were carried out for 60 min in water bath at 37 °C and the enzyme reactions stopped by the addition of 0.1 ml of 6 N sulfuric acid. The acidified reaction mixtures were assayed for acetolactate by decarboxylation at 70 °C for 5 min and subsequent measurement of the acetoin formed [11, 12], as follows: the quenched reaction samples were centrifugated at 9,800 × *g* for 3 min to remove protein, 1.6 ml of distilled water and 0.4 ml each of 0.5% (w/v) creatine and 5% (w/v) α-naphtol prepared in 2.5 N sodium hydroxide were added in rapid succession with immediate mixing to 0.4 ml supernatants. Then the mixtures were incubated at 70 °C with several intervening mixings to ensure efficient aeration (essential to pink color development). After 10 min, light absorbance at 530 nm was measured. The activity of control assay samples acidified prior to the addition of the enzyme solution ('standard assay control'), were subtracted from each sample.

The protein content of enzyme solutions was determined by the Coomassie Brilliant Blue binding assay of Bradford with bovine serum albumin serving as the standard [13].

Under these assay conditions, acetolactate formation is directly proportional to the protein concentration. ALS activity is expressed as nmol of acetoin formed per mg protein per h using a standard curve prepared with commercially available acetoin.

*In vivo* effect of IQ or MSM on ALS activity

Twenty-five corn seeds were germinated in darkness with a constant temperature of 28 °C on moist filter paper treated with IQ or MSM. The used concentrations were 0 and 400 nM for IQ and 0 and 40 nM for MSM. Three days after sowing, the seedlings were harvested and the roots and shoots were separated for ALS activity measurement immediately. These experiments were repeated two times, each with six replicates.

*In vitro* effect of IQ or MSM and NA or oxabetrinil on ALS activity

Corn seeds were germinated as above. Three days after sowing, the seedlings were harvested and the roots were separated for assays immediately. This experiment was repeated two times, each with two replicates.

*Safener experiments*

Each of the above types of experiment was repeated using NA or oxabetrinil-pretreated seeds. Pretreated seeds received the herbicide safener NA or oxabetrinil applied as a dust (10 or 2 g/kg seed weight, respectively) 7 d prior to the experiments. The seeds were shaken with a known amount of NA or oxabetrinil powder in a glass container for 60 min. The weight of the container before and after removal of the seeds was determined, so as to ensure as nearly as possible that each batch of seeds received the same amount of NA or oxabetrinil (1 or 0.2%, w/w, respectively). These amounts were the maximum amounts that could be applied with adequate adherence to the seeds.

## Results

*Inhibition of root and shoot lengths*

Shoot growth had a higher level of resistance to IQ and MSM than root growth (Table I). In the

Table I. Influence of herbicide concentrations on root and shoot lengths from corn seedlings.

| Treatment Compound | Rate [nM] | Length [mm] Roots | Shoots |
|---|---|---|---|
| None | | 62 | 23 |
| IQ | 40 | 61 | 24 |
| IQ | 100 | 56 | 29 |
| IQ | 400 | 38 | 28 |
| IQ | 1000 | 26 | 23 |
| MSM | 4 | 47 | 17 |
| MSM | 12 | 34 | 14 |
| MSM | 40 | 31 | 18 |

(Average of three trials)

Table II. Influence of herbicide treatment on root and shoot lengths from corn seedlings safened with NA or oxabetrinil.

| Treatment Safener | Herbicide | Length [mm] Roots | Shoots |
|---|---|---|---|
| None | None | 62 | 23 |
| None | 400 nM IQ | 38 | 28 |
| None | 40 nM MSM | 31 | 18 |
| NA | None | 68 | 36 |
| NA | 400 nM IQ | 35 | 34 |
| NA | 40 nM MSM | 31 | 32 |
| Oxabetrinil | None | 57 | 32 |
| Oxabetrinil | 400 nM IQ | 34 | 26 |
| Oxabetrinil | 40 nM MSM | 29 | 26 |

(Average of three trials)

roots, the $I_{50}$ (concentration of compound producing 50% inhibition) were approximately 400 and 40 nM for IQ and MSM, respectively.

Shoot lengths were increased by application of a concentration of 1% (w/w) NA or 0.2% (w/w) oxabetrinil. In addition, IQ and MSM blocked the growth of roots of unsafened seeds as well as safened seeds (Table II). The sensitivity of roots of NA-pretreated seedlings to IQ was similar to that of oxabetrinil-pretreated seedlings. The growth of shoots of safened seeds was resistant to 400 nM IQ and to 40 nM MSM, but the growth of shoots of unsafened seedlings on IQ did not differ from that of untreated seedlings. However, shoots of oxabetrinil-pretreated seedlings were less resistant to MSM than were shoots of NA-pretreated seedlings.

*In vivo inhibition of ALS activity*

The *in vivo* activity of ALS extracted from roots and shoots of corn seeds treated with IQ or MSM was similar to that of untreated controls. However, pretreatment of seeds with NA or oxabetrinil prior to germination caused an increase in the level of extractable ALS activity from shoots of untreated seedlings (Table III). Interestingly, the ALS activity from roots and shoots of NA-pretreated seedlings was also enhanced to a large degree (>40%) when the seedlings were germinated on 40 nM MSM, whereas ALS activities from oxabetrinil-pretreated seedlings were only enhanced to a lesser degree (≈20%). ALS activities from safened seedlings germinated on 400 nM IQ were unchanged.

Table III. Influence of herbicide treatment on extractable ALS activity from roots and shoots of corn seedlings unsafened or safened with NA or oxabetrinil.

| Treatment Safener | Herbicide | ALS (nmol acetoin/mg protein × h) Roots | Shoots |
|---|---|---|---|
| None | None | 159 | 280 |
| None | 400 nM IQ | 134 | 278 |
| None | 40 nM MSM | 163 | 259 |
| NA | None | 159 | 322 |
| NA | 400 nM IQ | 160 | 303 |
| NA | 40 nM MSM | 223 | 390 |
| Oxabetrinil | None | 147 | 338 |
| Oxabetrinil | 400 nM IQ | 156 | 265 |
| Oxabetrinil | 40 nM MSM | 191 | 312 |

(Average of six duplicate trials)

*In vitro inhibition of ALS activity*

The addition of IQ or MSM *in vitro* to ALS extracted from roots from unsafened or safened and herbicide treated seedlings resulted in a decrease in activity as compared with an IQ or MSM free control (Table IV). ALS activity from unsafened seedlings was inhibited 21% by 400 nM IQ and 70% by 40 nM MSM *in vitro*. Nevertheless, ALS activity from roots of seedlings germinated on 400 nM IQ was not inhibited by 400 nM IQ *in vitro*. Furthermore, ALS activity from roots of safened seedlings was less inhibited by IQ and MSM than that of unsafened seedlings.

## Discussion and Conclusion

IQ inhibited growth of combined corn roots and shoots but it was significantly less potent than MSM (Table I).

Pretreatment of corn seeds with NA or oxabetrinil did not significantly alter the amount of length injury from 400 nM IQ or 40 nM MSM (Table II). However, analyses showed that the root responses of the seedlings differed from the shoot responses. NA-pretreated seedlings also varied from oxabetrinil-pretreated seedlings in their response to the applied herbicide. The effective action of oxabetrinil on roots was decreased by oxabetrinil-caused reductions in root lengths without IQ or MSM treatment. Phytotoxic effects of corn safeners have been previously reported [10].

It has been reported that NA enhanced the activity of extractable ALS in pretreated corn seedlings [14]. This response was partially confirmed in our work (Table III). Both safeners prevented reductions in ALS activity caused by MSM treatments. NA was the most effective safener in this respect, preserving ALS activity in roots and shoots. Oxabetrinil pretreatment prevented ALS activity reduction in root tissues. The slight decrease in ALS activity following corn treatment with IQ documented by Barrett [10] was less evident in our experiments. IQ treatment slightly reduced only the extractable ALS activity of shoot tissues in unsafened and safened seedlings. Variations in experimental protocols, especially the use of different corn varieties, could account for this. Nevertheless, it is possible that IQ, coextracted from the shoots with the ALS, caused a slight *in vitro* enzyme inhibition. However, when the extraction medium contained 100 μM imazapyr, the ALS was not inhibited [7].

Although the data of Table IV are preliminary results, safener pretreatments plus herbicide treatments used herein appeared to alter *in vitro* ALS inhibition by 400 nM IQ or 40 nM MSM when the herbicides were included in the reaction mixture. Root ALS from safened seedlings was less sensitive to inhibition by the two herbicides than the enzyme from unsafened seedlings. Furthermore, the *in vitro* ALS activity inhibition in roots of the safened seedlings was nullified by the addition of 400 nM IQ to the growth buffer.

Table IV. *In vitro* ALS activity from roots of corn seedlings unsafened or safened with NA or oxabetrinil and herbicide treated during 3 days.

| Treatment | | ALS (nmol acetoin/mg protein × h) | |
|---|---|---|---|
| Safener | Herbicide | + 400 nM IQ | + 40 nM MSM |
| None | None | 122 | 44 |
| None | 400 nM IQ | 141 | 67 |
| None | 40 nM MSM | 130 | 83 |
| NA | None | 135 | 96 |
| NA | 400 nM IQ | 178 | 114 |
| NA | 40 nM MSM | 204 | 124 |
| Oxabetrinil | None | 105 | 73 |
| Oxabetrinil | 400 nM IQ | 150 | 96 |
| Oxabetrinil | 40 nM MSM | 158 | 77 |

(Average of two duplicate trials)

These studies reported a general but not precise correlation between inhibitory effects of herbicides and activity of safeners. The similarity of the two ALS inhibitors is interesting. Therefore, further research is needed to elucidate the exact biochemical and physiological mechanisms of action of NA and oxabetrinil. Future reports will describe our progress towards this end.

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