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Is There a Relationship Between Branched Amino Acid Pool Size and Cell Cycle Inhibition in Roots Treated with Imidazolinone Herbicides?

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Abstract. Excised pea root tips were cultured in White's medium for 24 h and then treated for 12 h with one of the imidazolinone herbicides at 0.2, 2, 20, or 200 μM. Pursuit and Assert were almost ineffective in inhibiting the mitotic index (MI), except at the highest concentrations. Arsenal (ARS) and Scepter both showed good inhibition with 20 μM by 8 h. Adding all three branched amino acids (BAA) (VAL, ILE, and LEU) at 0.1 mM blocked herbicide action. Treatment with the BAAs singly had no protective effect. Experiments were performed to determine the BAA pool size and MI after an 8-h treatment with ARS at 2 and 200 μM and Chlorsulfuron (CS), a sulfonylurea herbicide, at 28 nM. Both CS and ARS at 200 μM inhibited the MI to almost 0 by 8 h. ARS at 2 μM inhibited the MI by about 40%. The BAA pool size in all three treatments was reduced by approximately 50%, whether the MI was totally blocked or not. The 1-mm root tips had a greater amount of VAL than did the mature portions of the roots, whereas ILE and LEU were slightly less in the root tip. Other soluble amino acids did not show consistent differences between herbicide-treated roots and controls. The implications of the pool size reduction, in instances where the MI was not totally inhibited, is discussed in light of new data from other laboratories on the mode of action of the imidazolinone herbicides.

The sulfonylurea herbicides are known to inhibit the activity of acetolactate synthase, a key enzyme in the biosynthesis of the branched amino acids (BAAs)—VAL, ILE, and LEU (Ray 1982, 1984). One of the herbicides in this family, Chlorsulfuron (CS), selectively inhibits the cell cycle in root tips without apparently affecting any other metabolic process (Rost 1984). The cell cycle inhibitory effect can be blocked or reversed by adding BAAs to the culture medium (Robbins and Rost 1987, Rost and Reynolds 1985). Another family of herbicides, the imidazolinones, are also known to inhibit acetolactate synthase (acetohydroxyacid synthase) (Shaner et al. 1984). The action of both herbicides can be reversed or blocked by treating tissues with BAAs, and supposedly both have similar modes of action (Rost and Reynolds 1985, Shaner and Reider 1986). The general hypothesis for the mode of action was that these herbicides work by depleting the BAA pool size and thereby perhaps causing the depletion of cell-cycle-specific proteins or nucleic acids (Rost 1984, Shaner and Reider 1986). It has also been suggested that these herbicides might inhibit growth by the buildup of a toxic intermediate, such as 2-ketobutyrate (LaRossa et al. 1987, Rhodes et al. 1987), but this has been demonstrated only in bacterial cells.

In this study we tested four imidazolinone herbicides for cell cycle effects using cultured pea root tips. We also measured the BAA pool size to correlate soluble amino acid levels to cell cycle activity.

Materials and Methods

Pea seeds, *Pisum sativum* cv. Alaska, were sterilized in 10% household bleach and 0.75% (wt/vol) Alconox detergent for 5 min followed by a 70% ethanol rinse and two rinses in sterile distilled water. Seeds were sown in sterile vermiculite, covered with aluminum foil, and placed in the dark at 25°C for 4 days. The 1-cm terminal of 3–7-cm long roots was removed and placed in 500-ml culture flasks, containing 130 ml White's medium (White 1943) at pH 4.6 (adjusted prior to autoclaving). Fifty roots were placed in each flask. The roots were equilibrated for 24 h in the dark on an orbital shaker (90 rpm) at 25°C.

The explants were divided into five groups of two flasks each. One group, which served as the "zero time" control, was re-

moved and sampled immediately after the equilibration period. The remaining four groups were subcultured into fresh White's medium (pH 4.6) of the following composition: treatment 1, 2.0 μM Arsenal (ARS) (American Cyanamid Co., Princeton, NJ, USA); treatment 2, 200 μM ARS; treatment 3, 28 nM chlorsulfuron (CS) ("Glean," E. I. duPont de Nemours and Co., Wilmington, DE, USA); and treatment 4, White's medium control. The flasks were incubated an additional 8 h under the same conditions and then sampled for the mitotic index (MI) and prepared for soluble amino acid extraction.

The dose–response experiments were conducted in a similar way but using different concentrations (0.2–200 μM) of four different imidazolinone herbicides—Pursuit, Assert, ARS and Scepter. Reversal experiments were conducted with roots treated with Scepter or ARS at 20.0 μM, and only the Scepter data is shown. The following treatments were conducted: (1) White's medium control; (2) White's medium control containing VAL, ILE, and LEU at 0.1 mM; (3) 20 μM ARS or Scepter; (4) 20 μM ARS or Scepter plus 0.1 mM VAL, ILE, and LEU; (5) 20 μM ARS or Scepter plus 0.1 mM ILE; (6) 20 μM ARS or Scepter plus 0.1 mM LEU; and (7) 20 μM ARS or Scepter plus 0.1 mM VAL.

The technical samples of the herbicides were prepared as follows. ARS, 2-[4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]-nicotinic acid (MW 261.3), was dissolved first in 1 ml of 1.0 N KOH, then diluted very slowly with distilled water to stock concentration. The other imidazolinone herbicides were treated in the same manner. CS, 2-chloro-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]-benzene sulfonamide (MW 357.78), was dissolved in 3 ml of 100% acetone, then diluted very slowly with distilled water to stock concentration. Herbicide stock solutions were sterilized with 0.22 μm Membra-Fil cellulose ester filters (Nucleopore Corp., Toronto, Canada) prior to being added to the sterile culture medium.

Mitotic Index Determination

Five root tips were sampled at 0 and 8 h and fixed in absolute ethanol:glacial acetic acid (3:1) overnight. Following fixation, the root tips were hydrolyzed for 20 min in 5 N HCl and stained with Schiff's reagent (Fisher Scientific, San Francisco, CA, USA) for a minimun of 2 h. The 2-mm terminal of each stained root tip was excised, placed on a glass slide in a drop of 45% acetic acid, and macerated with the rounded end of a glass rod. A cover glass was placed over the tissue, and the slide was pressed between blotter papers to spread the cells. The cells were affixed by placing the slide on a block of dry ice for several minutes, popping off the cover glass, and then plunging the slide into absolute ethanol. The slides were made permanent with Euparol (ASCO Laboratories, Gorton, Manchester, UK) and a fresh cover glass. The slides (five per treatment) were scored for MI by counting the number of mitotic figures per 500 cells and expressing this as a percentage. The standard errors of the mean (SEM) are shown in the graphs. All graphs and statistics were prepared using Cricket Graph and MacDraw II software.

Extraction of Soluble Amino Acids

The remaining roots (45 per flask) from each treatment were rinsed in water, flash frozen with liquid nitrogen, and lyophilized for 48 h (VirTis Model 6 Bench Top Freeze Dryer, VirTis Co., Gardiner, NY, USA). The dried roots from each treatment were ground together with mortar and pestle and extracted three times with 3 ml of 80% ethanol at 80°C for 1 h. The ethanol solution was separated from the macerated root tissue by centrifugation, and the pooled and filtered supernatants were taken to dryness in a warm water bath using nitrogen gas. The dried samples were analyzed for soluble amino acid content with a Beckman 6300 Amino Acid Analyzer (Beckman Ind., Palo Alto, CA, USA) using lithium citrate buffers.

Nelson 2600 chromatography software (Nelson Co., Cupertino, CA, USA) was utilized for data processing. These experiments were repeated five times; the histograms show one representative experiment.

Results

Dose–Response and Reversal

Four imidazolinone herbicides were tested at four concentrations (0.2, 2, 20, and 200 μM). Pursuit was relatively ineffective, showing only partial inhibition of the MI by 12 h at 200 μM (Fig. 1A). Pea meristems were even more resistant to Assert, where at 200 μM the MI was inhibited by just over 50% after 12 h (Fig. 1B). ARS was a better inhibitor, with about 60% inhibition with 20 μM after 8 h, and total inhibition by 8 h with 200 μM (Fig. 1C). Scepter was equally effective with good inhibition of the MI with 20 and 200 μM treatments by 8 h (Fig. 1D).

Adding BAAs to the culture medium is known to protect meristems from MI inhibition by CS (Rost and Reynolds 1985). Since the imidazolinone herbicides are known to also inhibit BAA synthesis, we wanted to determine if adding BAAs singly or in combination would protect root tips from herbicide effects. Roots were treated with Scepter or ARS at 20 μM with and without BAAs (Fig. 2; ARS data is not shown). The untreated controls with and without the three BAAs showed a constant MI, whereas the 20 μM treatment and the herbicide plus single BAA-supplemented treatments showed inhibition. If all three BAAs were added to the medium with herbicide, total protection occurred. Apparently, the imidazolinones and the sulfonylureas inhibit the cell cycle in a similar way but with a different dose requirement.

BAA Levels

In these experiments, roots were cultured for 24 h after excision, then treated with 2 or 200 μM ARS or 28 nM CS, and then sampled for MI and soluble BAA amounts (Fig. 3). The MI was constant between the 0 and 8 h control, was reduced by about 50% with 2 μM ARS, and was almost totally inhibited with 200 μM ARS or 28 nM CS.

Note that the BAA levels were reduced in the 8 h

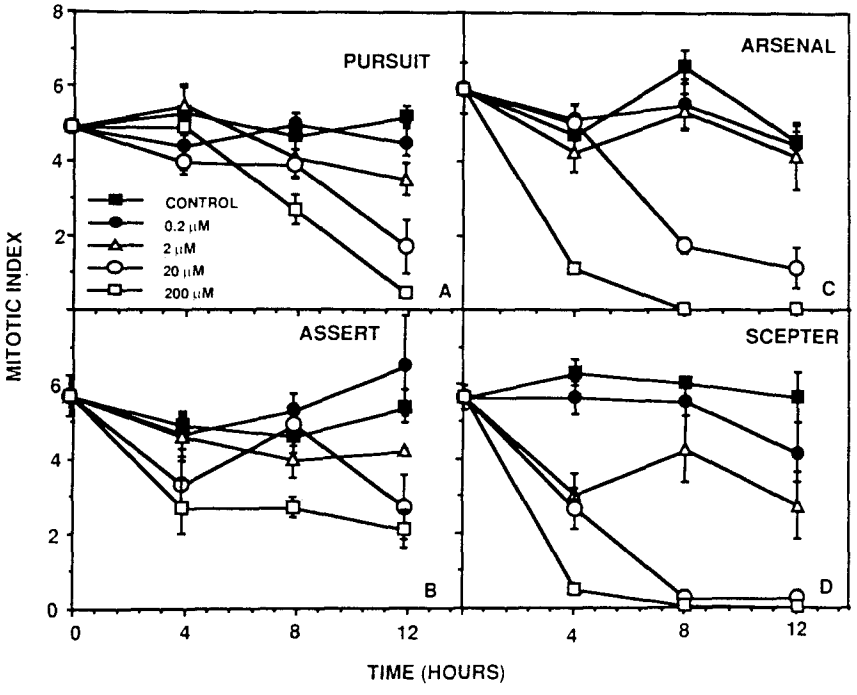

Fig. 1. Dose–response curves for four different imidazolinone herbicides showing MI in root tips plotted against time of treatment. Roots were excised and grown in White's medium for 24 h before herbicide was added. Vertical bars in each graph represent the SEM.

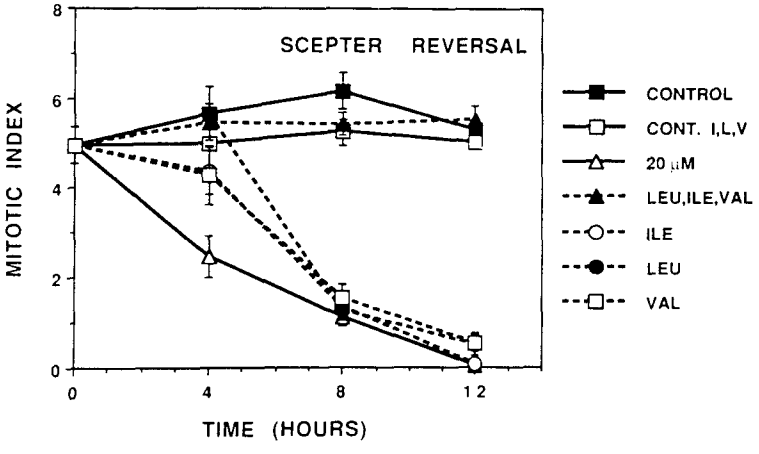

Fig. 2. Curves of Scepter-treated roots with co-treatment with all three or single treatments of BAAs.

control. This was a consistent observation among experiments. Note also that the levels of all three BAAs were only slightly reduced compared to the 8 h control, but were depressed almost equally in all three herbicide treatments. This is noteworthy, because the lower ARS concentration showed a 50% reduction of the MI, whereas the higher concentration and the CS treatment showed complete inhibition. This observation suggests that a reduction in the BAA pool size by itself cannot explain the reduction in the MI.

Next we treated roots with ARS at 2 and 200 μM and then separated the 1-mm tips from the mature portion of the cultured root. The MI was determined from the tips, and both were examined for BAA amounts (Fig. 4). This was done to determine whether BAAs were possibly sequestered in the mature part of the roots, which would mask the inhibition of synthesis in the meristem. The only evidence of uneven distribution was that VAL was found in much higher amounts in the 1-mm meristems than in the mature root segment. Also, ILE and LEU tended to be slightly less concentrated in the meristems than in the mature tissues. Again in this experiment, the higher concentration of ARS and 28 nM CS totally inhibited the MI, the lower concentration of ARS showed partial inhibition, but all three showed similar levels of BAAs.

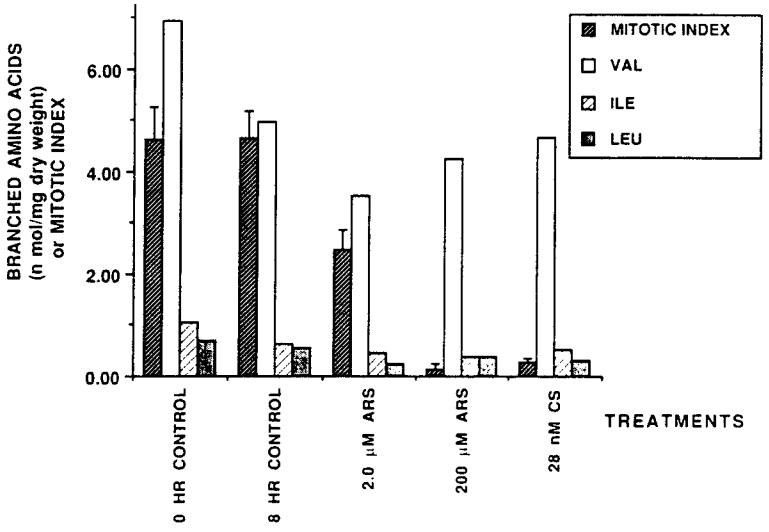

Fig. 3. Histogram of amounts of soluble BAAs in root tips compared to MI in roots treated with ARS or CS for 8 h.

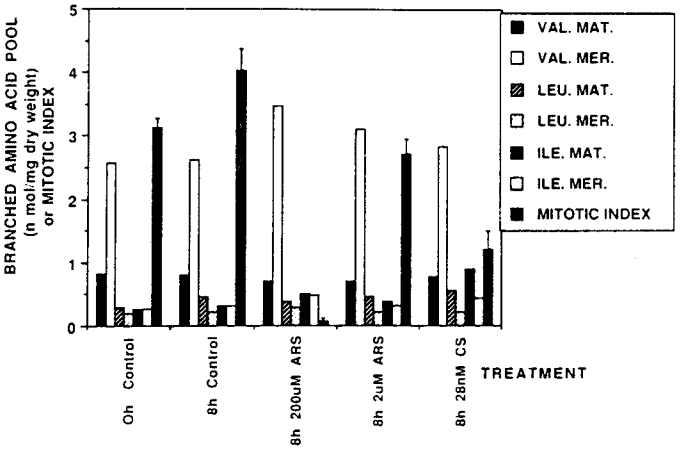

Fig. 4. Histogram of soluble amino acid levels in root tip meristems (MER) and mature portions (MAT) of roots compared to MI after 8-h treatment.

We also examined the levels of the other amino acids after an 8-h treatment with ARS at 2 and 200 µM and CS at 28 nM. Expressed as percent of the 8-h control, we observed only minor differences between treatments. GLU was consistently higher in the herbicide-treated roots (Fig. 5).

Discussion

The sulfonylurea and imidazolinone herbicides are broad spectrum herbicides that selectively inhibit growth events, especially the progression of cells through the cell cycle (Ray 1982, Rost 1984, Shaner et al. 1984). These chemicals are rather unique among herbicides, in that their mode of action was thought to be clearly understood. The original model for the sulfonylurea herbicides (Ray 1982) was that the herbicide inhibited the enzyme acetolactate synthase which in turn blocked BAA biosynthesis. The resulting BAA pool size decrease supposedly inhibited the synthesis of certain specific proteins which then inhibited cell cycle progression quite specifically.

Shaner and Reider (1986) worked with corn suspension cultures and showed a 70–90% decrease in BAA levels with ARS. Shaner et al. (1985) working with Pursuit showed no effect on protein synthesis but a 31% decrease in free BAA levels. Shaner et al. (1984) showed that BAA levels drop after treatment with imidazolinone herbicides and that ILE, VAL, and LEU were needed for complete reversal. They suggested again that the mode of action of these chemicals involved the specific inhibition of certain protein syntheses.

Herbicide workers were very excited about these two groups of herbicides, from both a technical and practical view, because the reactions they inhibited were present only in plant cells, and because they represented the only chemicals for which a clear

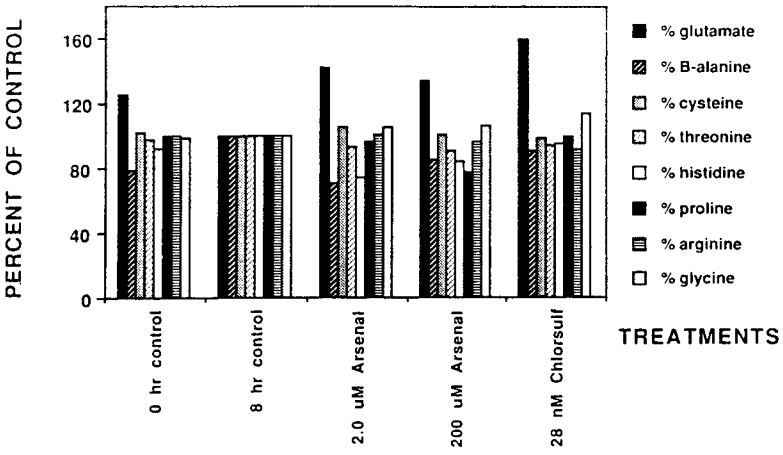

Fig. 5. Levels of different soluble amino acids in cultured roots (control) and roots treated with ARS or CS for 8 h.

mode of action was understood. Problems with the model did not surface until LaRossa et al. (1987) suggested that the build-up of the intermediate 2-ketobutyrate (2-KB) caused the inhibition.

According to LaRossa et al. (1987) and Van Dyk and LaRossa (1989), *Salmonella typhimurium* cells accumulated 2-KB after being treated with a sulfonylurea herbicide. Adding ILE to inhibited cultures reversed the accumulation of 2-KB by feedback inhibition of the enzyme threonine deaminase. These results suggested that it is not the depletion of BAA, but rather the accumulation of 2-KB that terminated growth of these bacterial cells. LaRossa et al. (1987) further suggested that this accumulation induces several secondary responses, including the inhibition of acetyl-CoA synthesis, which in turn inhibits growth events. To date, no published studies have appeared which test this new hypothesis in higher plants, but the results of our study and other clues provide indirect evidence that it may be true. Rhodes et al. (1987), in fact, may have been on the right track by demonstrating that α-amino-n-butyrate, another BAA intermediate, accumulated after treating *Lemna* plants with CS. Coincidently, several animal and a few plant cell biologists have recently been examining the effects of butyrate on the regulation of the cell cycle. This compound is a metabolic byproduct of 2-KB. It is known to inhibit cell division in *Allium* root tips, but at a high concentration—4–6 mM (Langzagorta et al. 1988). It has also been shown to acetylate histones in *Physarum* (Loidl et al. 1984) and to acidify the cytoplasm and thereby stop cell division in *Hordeum* root tips (Reid et al. 1985).

Our results show that two different doses of ARS, one which inhibits the cell cycle and one which only partially inhibits it, both reduce the BAA pool by less than 50%. This observation has led us to conclude that the BAA pool reduction in itself is not the cell cycle inhibition step.

This is not to suggest that a reduction in amino acid levels cannot alter protein synthesis and the character of protein populations. Brunschede and Bremer (1971), for example, showed that amino acid starvation caused the rate of protein synthesis to drop within a few minutes and that the composition of proteins changed. In bacteria, protein synthesis is shut down when amino acid levels get below a certain critical level. This is the so called "stringent response" (Lewin 1987). In root tips, determining a specific role of amino acid starvation is a complicated problem because of the compounding effect of amino acids available from protein degradation, which may be high enough to continously maintain amino acid levels for a time.

Shaner and Reider (1986) suggest that a short-lived protein may be involved in the regulation of S and M progression. This may still be true, it certainly is an attractive model, but it seems now that this protein probably doesn't have a specific BAA requirment, but instead may be specifically inhibited by α-amino-n-butyrate, α-ketobutyrate, or some other BAA intermediate.

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