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Interactions and Effects on Cysteine Synthase Activity of Aminooxyacetate and Boc-Aminooxyacetate on the Bioherbicides *Colletotrichum truncatum* and *Alternaria cassia* and Their Weed Hosts

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Abstract

Aminooxyacetate (AOA) is a pyridoxal phosphate antagonist that inhibits various plant enzymes (including transaminases) which require pyridoxal phosphate as a cofactor and it exhibits phytotoxic and herbicidal properties. We examined AOA and its analog, *N-t*-butoxycarbonyl-AOA (Boc-AOA) for phytotoxicity, interactions with weed pathogens (bioherbicides), and effects on an important pyridoxal requiring enzyme, cysteine synthase (CS, E.C. 4.2.99.8). Studies were performed on two weeds, *i.e.*, hemp sesbania [*Sesbania exaltata* (Raf.) Rybd. Ex A.W. Hill] and sicklepod (*Senna obtusifolia*), and two pathogens, (*Colletotrichum truncatum* and *Alternaria cassiae*), that are bioherbicidal agents against hemp sesbania and sicklepod, respectively. Pathogenicity tests, and assays for extractable, and *in vitro* CS activities were utilized. Phytotoxicity bioassays indicated that the bulky *t*-butoxycarbonyl moiety substitution on the AOA molecule did not substantially hinder expression of biological activity of Boc-AOA in these tests. Generally, spray application of the compounds to young dark-grown seedlings caused little growth effects, but root-feeding of the chemicals reduced growth (stem elongation) in both weeds. Hemp sesbania was generally more tolerant than sicklepod to these compounds. The only apparent positive interaction of the chemicals with these pathogens was the Boc-AOA: *C. truncatum* combination treatment on hemp sesbania. Both compounds reduced extractable CS in the seedlings by 30%, 72 h after treatment. CS activity was reduced by 15% in hemp sesbania

treated with *C. truncatum* but increased 20% above control levels after infection of sicklepod by *A. cassiae*. This latter effect suggests that CS may be involved in sicklepod defense mechanisms against this pathogen.

Keywords

Aminooxyacetate, Bioherbicide, Cysteine Synthase,
Pyridoxal Phosphate Antagonist, Sicklepod, *Senna obtusifolia,* Hemp Sesbania, *Sesbania exaltata*, Transaminase

1. Introduction

Aminooxyacetate (AOA) (**Figure 1**) was patented as a herbicide in 1964 [1]. This compound is an *in vitro* inhibitor of phenylalanine ammonia-lyase (PAL), phenylalanine transaminase [2], other transaminases [3] [4], and ethylene production in plants [5]. In a major study, AOA was found to be a potent inhibitor of all plant transaminases tested [6]. AOA inhibits alanine aminotransferase competitively with amino acid substrates and non-competitively with oxo-acid substrates [7] [8]. AOA acts as a pyridoxyl phosphate antagonist, and many plant transaminases are inhibited by such antagonists including, semicarbazide, hydroxylamine, and cyanide. In soybean [*Glycine max* (L.) Merr.] seedlings, root-fed AOA has been shown to inhibit growth, and to reduce anthocyanin, activity is competitively inhibited by AOA with amino acid substrates and non-chlorophyll, and extractable PAL levels [9]. Recently, an analog of AOA, *N*-*t*-butoxycarbonyl-AOA (Boc-AOA) (**Figure 1**) became available, but little or no information on the biological activity of this new compound has been published.

For several decades there has been considerable interest in using plant pathogens for biological weed control [10]-[16]. Although a large number of pathogens have been discovered to infect various weeds, many of these pathogens have insufficient virulence to be useful in bioherbicide programs. Some herbicides and pathogens interact synergistically, resulting in increased weed control efficacy [17]. If such interactions are sufficiently potent, lower concentrations of herbicides and pathogen propagules may be adequate to achieve weed control. Also plant defense may be intimately related to synergistic plant: pathogen interactions. Plant defense mechanisms are important to protect plant from attack by microorganisms, pathogens and other stress factors. Although some biochemical defenses in plants have been elucidated, nearly all of this information relates to crop plants. Only in a few instances has the biochemistry of pathogen interactions with weeds and the weed defense responses been investigated [13]. Studies of AOA effects on certain pathogen: plant interactions have shown that this compound can lower plant defense and increase disease susceptibility in plants, *i.e.*, tobacco mosaic virus in tobacco [18], *Fusarium oxysporum* in tomato [19], and *Puccinia coronata* in oat [20]. These effects were generally attributed

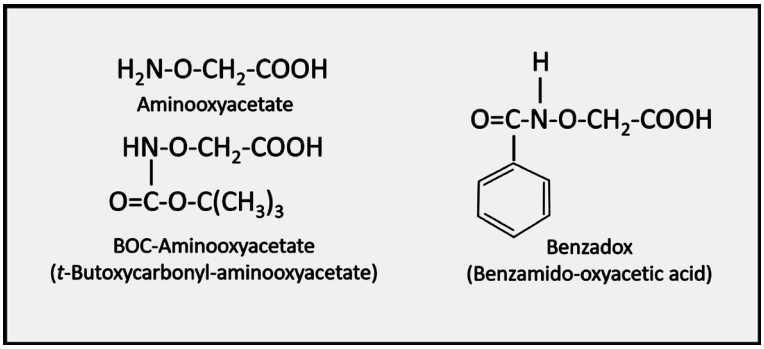

Figure 1. Structural comparison of aminooxyacetate (AOA) and the analogs *N*-*t*-butoxycarbonyl-aminooxyacetate (Boc-AOA) and benzadox.

to the inhibition of PAL activity and/or concomitant reduction of phytoalexin synthesis. However as previously stated, AOA has effects on many transaminases and other biochemical processes that might also be related to plant defense. Furthermore, AOA effects on weed pathogen interactions have not been studied, and there is little or no biological data on Boc-AOA.

Higher plants and certain algae are the only eukaryotes that assimilate inorganic sulfur into organic compounds. Incorporation of inorganic sulfur into cysteine via cysteine synthase (CS, E.C. 4.2.99.8) (also called O-acetylserine sulfhydrylase) is the final step in sulfate reduction/assimilation in plants as outlined [21]. Cysteine is synthesized from serine in two steps; serine transferase catalyzes acetylation of serine using acetyl-CoA to yield O-acetylserine, and then CS catalyzes addition of inorganic sulfide to O-acetylserine with release of acetate and cysteine. CS has been deemed essential for plant growth [22] and has been isolated from *Datura* [23] and *Brassica* species [24] [25] and many other plants. The interactions of herbicides and safeners on CS as a potential target for new herbicidal compounds has also been examined [21] [26] [27] [28] [29]. CS has also been shown to be inhibited by AOA [28]. Since CS requires pyridoxal phosphate as a cofactor, the enzyme is a possible sensitive site of AOA and Boc-AOA. Thus, it was also possible that these aminooxy-compounds might interact with these bioherbicides in a synergistic manner.

To test these hypotheses, we choose the following objectives: 1) to compare the phytotoxicity of AOA and Boc-AOA (**Figure 1**) on seedlings of two economically important weeds, hemp sesbania [*Sesbania exaltata* (Raf.) Rybd. ex A.W. Hill] and sicklepod *Senna obtusifolia* (L.) Irwin and Barnaby], 2) to evaluate these compounds as *in vivo* and *in vitro* inhibitors of CS in these weed seedlings, and 3) to examine possible interactions of the compounds with two important biocontrol pathogens {*Collectotrichum truncatum* (Schwein.) Andrus and W.D. Moore on hemp sesbania [[30] Boyette 1991] and *Alternaria cassiae* Jurair and Khan on sicklepod [31] [32]. Both of these weeds have been listed in surveys as very troublesome in several crops [33]. These pathogens were chosen as test organisms since they were both discovered at our laboratory and because they and their weed hosts have been used to develop seedling bioassays to test

the phytotoxicity of bioherbicides (pathogens and microbial products) and other compounds [34].

2. Materials and Methods

2.1. Weed Seeds and Seedling Propagation

Hemp sesbania [*Sesbania exaltata* (Raf.) Rybd. ex A.W. Hill] and sicklepod [*Senna obtusifolia* (L.) Irwin and Barnaby] seeds were harvested from weed plots grown at the Crop Production Systems Research Unit, Stoneville, MS. Seeds were mechanically scarified, planted in paper towel cylinders, and grown hydroponically in the dark as described previously [34]. After 96 h growth, uniform seedlings were selected for test, and again placed in paper towel cylinders. Then seedlings were used in hydroponic bioassays [34], after treatment with chemicals, pathogen spores, or the combination of chemical and spores.

2.2. Pathogen Spore Production

Cultures of *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore and *Alternaria cassiae* Jurair and Khan are pathogens of hemp sesbania and sicklepod, respectively. Cultures of these fungi from isolates discovered and maintained in our laboratory at Stoneville were propagated on potato dextrose agar as described elsewhere [32] [30] in Petri dishes and the spores produced were collected by brushing from the surface of the colonies. Spores were suspended in deionized water and diluted (using a hemocytometer) to achieve concentrations of 1.0×10^5 and 1.5×10^5 spores ml^{-1} for *A. cassiae* and *C. truncatum*, respectively. These spores were used directly for the preparation of spray inoculum for application to the seedlings.

2.3. Plant Growth and Application of Chemicals and Bioherbicides

Aminooxyacetate (AOA) and Boc-AOA, each at 1.0 mM, were applied (hand-held sprayer or root-fed) to 96 h-old, dark-grown hemp sesbania and sicklepod seedlings in paper towel cylinders as described above. Control seedlings received only a water spray. Shoot elongation in continuous darkness was measure after an additional 72 h after treatment. AOA and Boc-AOA were also applied to seedlings, alone or combined with the *C. truncatum* or *A. cassiae* for testing possible interactions. Each replication consisted of a minimum of four seedlings and each treatment was triplicated.

2.4. Chemical Sources

All reagent grade quality chemicals were used and were obtained from Sigma Chemical Co., St. Louis, MO.

2.5. Cysteine Synthase Assay

After removal from the dark growth chamber 72 h following treatment, plant seedling shoot tissue was homogenized in an electric blender with 200 mM po-

tassium phosphate buffer (pH 7.8), containing 1.0 mM dithiothreitol, and 1.0 mM ethylenediamine-tetra-acetic acid (EDTA). Assay mixtures contained 200 mM potassium phosphate buffer (pH 7.8), O-acetyl-L-serine(5 mM), sodium sulfate (1.0 mM), pyridoxyl-phosphate (0.05 mM), dithiothreitol (1.0 mM) and enzyme extract in a total of 1.0 ml. Reactions were run at 25˚C and were terminated with 2.0 ml HCl:glacial acetic acid (1:1, v:v) as outlined previously [27]. Extractable activity of cysteine synthase was determined spectrophotometrically (A_{560}), based on formation of the ninhydrin-cysteine reaction complex [35]. Enzyme activity values were based on a standard curve of absorbance of the ninhydrin-cysteine complex formed from the reaction of various concentrations of cysteine. Protein in enzyme extracts was determined using the Bradford reagent [36]. Each extraction utilized 4 to 6 seedlings per treatment and extractions of each treatment were performed in triplicate.

2.6. Greening Protocol and Chlorophyll Determination

Excised cotyledons of 96 h –old, dark-grown seedlings were placed in solutions of AOA, Boc-AOA, or water (control) in plastic well-plates (16 mm wide, 18 mm deep) for 2 h in the dark. The plates were then transferred to low light (90 - 100 $\mu E \cdot m^{-2} \cdot s^{-1}$, supplied by incandescent and florescent lightbulbs) for 72 h, and chlorophyll that accumulated in the greening excised cotyledons was determined using dimethyl sulfoxide (DMSO) extraction and spectrophotometry [37]. Each well contained a minimum of 4 cotyledon pairs, and each treatment was performed in triplicate.

2.7. Statistical Analyses

The experiment was set up as a randomized complete block, all treatments were performed in triplicate and the experiment was repeated. Data were analyzed and compared using analysis of variance at the P = 0.05 level. Significant differences were detected using the F-test and means were separated (Fisher's protected LSD at 0.05).

3. Results and Discussion

3.1. Phytotoxicity

Root-fed AOA inhibited shoot elongation in hemp sesbania slightly more than Boc-AOA up to 72 h after treatment. Both AOA and Boc-AOA were equally inhibitory to sicklepod shoot growth (**Figure 2**). There was little or no effect of foliar application of AOA or Boc-AOA on hemp sesbania shoot elongation (**Figure 3**), but both compounds reduced shoot elongation by 20% to 25% in sicklepod (**Figure 4**). The reduction of growth in hemp sesbania was not altered when *C. truncatum* and AOA were applied simultaneously (**Figure 3**). However, Boc-AOA combined with *C. truncatum* did produce a reduction in elongation of hemp sesbania that was significantly greater than that of the pathogen alone or the pathogen combined with AOA (**Figure 3**). The reason(s) for this differential

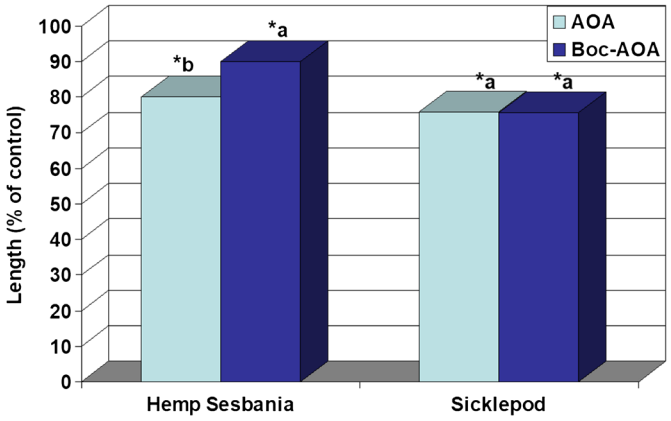

Figure 2. Effects of AOA and Boc-AOA each at 1.0 mM on shoot elongation of hydroponically dark-grown hemp sesbania and sicklepod seedlings, 72 h after root-feeding treatment under continuous dark growth conditions. Histogram bars within each species with the same letter are not different at p = 0.05 according to Fisher's LSD (P = 0.05).

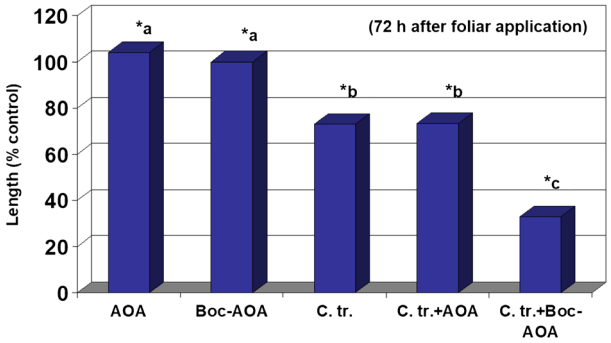

Figure 3. Effects of AOA and Boc-AOA each at 1.0 mM and *Colletotrichum truncatum* alone and combined with AOA or Boc-AOA on shoot elongation of hemp sesbania seedlings, 72 h after foliar application under continuous dark growth conditions. Histogram bars with the same letter are not different at p = 0.05 according to Fisher's LSD (P = 0.05).

effect of the two compounds are presently unexplainable. In sicklepod seedlings, treatments of the chemicals alone, pathogen (*A. cassiae*) alone, and the combination of pathogen plus AOA all caused similar growth reductions, *i.e.*, 20% to 25% below control levels (**Figure 4**). Boc-AOA plus *A. cassiae* caused a growth reduction of ca. 30% compared to control seedlings and was also slightly greater than the AOA plus pathogen treatment. In hemp sesbania and sicklepod, total chlorophyll content of greening cotyledons was inhibited by the compounds, but levels of this pigment were reduced to a greater extent in sicklepod than in hemp sesbania (**Figure 5**). Furthermore, the chlorophyll inhibition was greater for AOA than Boc-AOA in hemp sesbania, but greater for Boc-AOA than AOA in sicklepod.

3.2. Effects on Cysteine Synthase

AOA and Boc-AOA reduced extractable CS activity by 30% in seedlings of both weed species, 72 h after treatment (**Figure 6**).

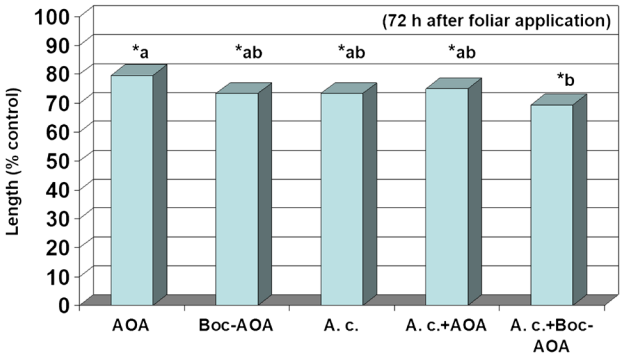

Figure 4. Effects of AOA and Boc-AOA each at 1.0 mM and *Alternaria cassiae* alone and combined with AOA or Boc-AOA on shoot elongation of hydroponically dark-grown sicklepod seedlings, 72 h after foliar application under continuous dark growth conditions. Histogram bars with the same letter are not different at p = 0.05 according to Fisher's LSD (P = 0.05).

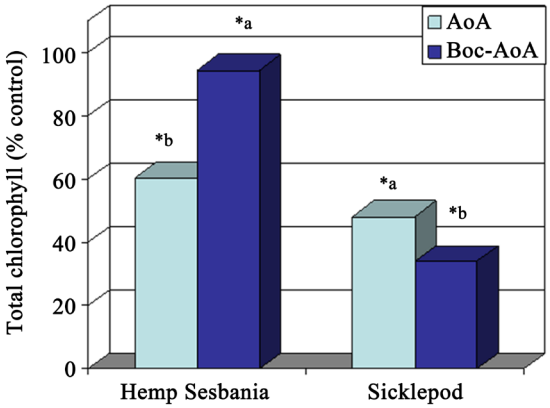

Figure 5. Effects of AOA and Boc-AOA on chlorophyll accumulation in greening excised cotyledons of etiolated hemp sesbania and sicklepod seedlings. Excised tissue was exposed to each compound at 1.0 mM followed be exposure to light for 72 h as described in the Materials and Methods. Histogram bars within each species with the same letter are not different at p = 0.05 according to Fisher's LSD (P = 0.05).

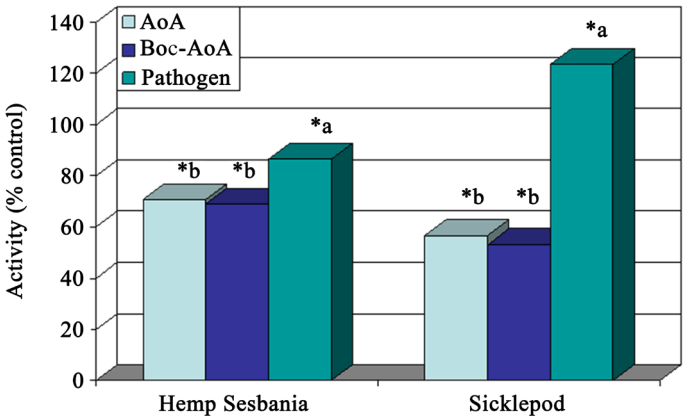

Figure 6. Cysteine synthase activity in hemp sesbania and sicklepod seedlings as affected by AOA, Boc-AOA, *Collectotrichum truncatum* or *Alternaria cassiae*. Histogram bars within each species with the same letter are not different at p = 0.05 according to Fisher's LSD (P = 0.05).

C. truncatum infection in hemp sesbania also lowered CS activity (15%), but *A. cassiae* infection in sicklepod increased activity 20% above untreated control levels. *In vitro* enzyme assays resulted in equal inhibition by 15% when either chemical and the substrates were added simultaneously to the enzyme (Figure 7). When either inhibitor was pre-incubated (10 min. at 25˚C) with enzyme without substrate, and then followed by substrate addition and assay, inhibition by Boc-AOA was not increased to any measurable extent, whereas the inhibition by AOA increased by an additional 20%.

Overall results indicate that hemp sesbania was generally more resistant than sicklepod to the phytotoxicity of AOA and Boc-AOA under these continuous dark-growth conditions and the greening of excised cotyledon test. The fact that these compounds had little effect after foliar application may be due to the waxy characteristics of these seedlings that could have slowed absorption of the compounds into the cytoplasm. Since cysteine synthase was inhibited *in vivo* and *in vitro* by AOA and Boc-AOA, and because there was no major difference in phytotoxicity of the two compounds in these young plants, apparently the *N-t*-butoxycarbonyl group does not alter the activity of the AOA molecule. An exception however, was the greater growth reduction caused by Boc-AOA plus *A. cassiae* compared to the AOA plus *A. cassiae* treatment. Furthermore, these chemicals had no apparent interactions with *C. truncatum* and *A. cassiae* efficacy on their respective weed hosts. The increase in extractable cysteine synthase activity in sicklepod following infection by *A. cassiae*, may be related to defense mechanisms of this weed against this pathogen.

There may be some inherent differences in the overall action of AOA and Boc-AOA, but it is not possible to tell from these results and the scope of this study. It is also not known if Boc-AOA is converted to AOA by enzyme hydrolysis of the substituted amide bond by these plants and their respective pathogens. A herbicidal analog of AOA, benzadox, is thought to be metabolized *in planta* to form AOA [38]. Also interesting is the naturally occurring compound irpexil [methyl-2-(acetamidooxy)acetate], a herbicidal compound produced by the basidiomycete *Irpex pachyodon* [39]. This compound has structural features analogous

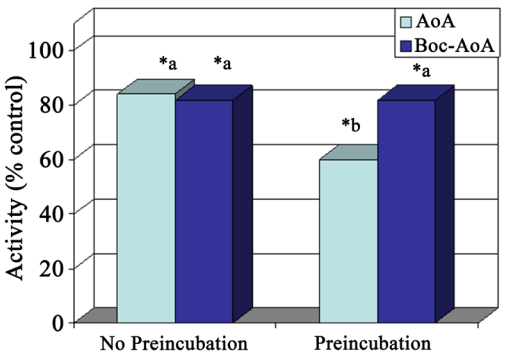

Figure 7. Effects of pre-incubation of AOA and Boc-AOA *in vitro* with cysteine synthase on enzyme activity. Histogram bars within each treatment with the same letter are not different at p = 0.05 according to Fisher's LSD (P = 0.05).

to AOA and the herbicide benzadox (**Figure 1**). Benzadox is an inhibitor of alanine aminotransferase [38], but the mechanism of action of irpexil, is unknown. Benzadox has been shown to be metabolized in plants to produce AOA [4]. Similar compounds such as aryl-substituted α-aminooxycarboxylic acids are also phytotoxic and act as auxin transport inhibitors [40]. AOA has been tested as a synergist with *C. coccodes* a bioherbicide of velvetleaf (*Abutilon theophrasti*) [41]. However, AOA was found to be inhibitory to the growth of this fungus on PDA in Petri dishes, when applied together with the fungus, or if applied via vacuum infiltration on velvetleaf leaves and furthermore, no synergy was observed in those studies.

Although the interactions that we found during these experiments resulted in no major synergistic effects, novel information was generated on the important enzyme CS and on AOA interactions with two bioherbicides that have received major attention as weed control agents.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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