Comparative Study on Artemisinin, 2,4-D, and Glyphosate

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Artemisinin is an antimalarial agent isolated from *Artemisia annua*. Recently, it has been demonstrated that this allelochemical is a selective phytotoxin. A comparative study was made between artemisinin and two commonly used herbicides, 2,4-D and glyphosate. At 5 μM all these compounds inhibited root induction in mung bean (*Phaseolus aureus*) seedling cuttings. Artemisinin exerted the same level of inhibition in growth as glyphosate. However, at the enzymatic level, artemisinin, unlike 2,4-D and glyphosate, inhibited peroxidase synthesis and showed no direct effect on tyrosinase activity. Both 2,4-D and glyphosate showed direct inhibitory effect on tyrosinase activity. Only 2,4-D increased to a high level the synthesis and secretion of peroxidase.

INTRODUCTION

Artemisinin was first introduced as an antimalarial agent in China (Klayman, 1985; Luo and Shen, 1987). Additional interest in this compound has developed because of its herbicidal activity (Chen et al., 1987; Chen and Leather, 1990; Duke et al., 1987, 1988). Other recent discoveries of plant allelochemicals with potential as herbicides have been reviewed (Putnam, 1988; Cutler, 1988; Duke and Lydon, 1987; Duke et al., 1988).

We report here the biological activity of artemisinin when tested and compared with commonly used herbicides, 2,4-D [(2,4-dichlorophenoxy)acetic acid] and glyphosate [*N*-(phosphonomethyl)glycine]. Their effects on growth and root induction in mung bean (*Phaseolus aureus*) seedling cuttings in vitro were investigated. The mechanism of action of glyphosate has been reported by a number of investigators (Becerril et al., 1989; Cole, 1985; Jaworski et al., 1984). Evidence has shown that glyphosate affected the metabolism of aromatic amino acids and the accumulation of shikimate pathway products. Peroxidase can influence a great array of physiological processes involved in growth, differentiation, lignin synthesis, and resistance to diseases in plants (Gaspar et al., 1982). Some molecular aspects of peroxidase synthesis in plants have been discussed (van Huystee, 1987). We also studied the effects of these three chemicals on peroxidase and tyrosinase to determine whether they have the same modes of action.

MATERIALS AND METHODS

Glyphosate, technical grade, was obtained from Monsanto Co.; (2,4-dichlorophenoxy)acetic acid (2,4-D) was purchased from Sigma Chemical Co. (St. Louis, MO). Artemisinin was purchased from Polyscience, Inc. (Warrington, PA). All the chemicals were freshly prepared before use.

Mung bean seeds (Carolina Biological Supply Co., Burlington, NC) were germinated in bale-compressed Baccto Grower's mix (Michigan Peat Co., Houston, TX) in a greenhouse. Seedlings (7–10 days old) used in this study were about 12–15 cm in height, and each one showed only its first pair of leaves. Immediately before experimentation, seedlings were excised about 1.5 cm from the soil surface with alcohol-sterilized scalpels. All the excised seedlings were washed extensively with lukewarm tap water and rinsed with sterilized distilled water before use. Various amounts of each test compound in 10 μL were delivered to 15 × 100 mm test tubes. Ten milliliters of sterile distilled water was then added to give final concentrations of 0, 5, 10, and 20 μM for each compound. All the seedlings were cut to 9.5 cm from the tip and inserted through the aluminum foil covering the test tubes.

Each treatment contained six replications, and the experiments were repeated. The seedlings were maintained in a growth chamber at 23–24 °C under constant light (236 $\mu E\ m^{-2}\ s^{-1}$) for 1 week. The numbers of roots were counted, and the fresh weight of stem tissues was measured. Dry weights of stem tissues were determined with an analytical balance after air-drying at 25 °C for 24 h. The detailed procedures of bioassay were similar to those described by Chen and Leather (1990).

To determine the time required for inhibition of root induction, the mung bean seedlings were treated as above with 10 μM compound. On days 1, 2, 4, and 7, the seedlings were removed from the treatment medium, washed with distilled water, and placed in fresh medium without the compound and incubated as described above. For time 0, the seedlings were placed immediately into medium without compound.

For enzymatic analyses, the tissues were washed and blot-dried, weighed separately, and then extracted with phosphate buffer, 0.05 M, pH 6 (4× v/w). All the extracts were centrifuged at 5000*g* for 10 min in the cold, and the supernatant was used for enzymatic determination. Peroxidase and tyrosinase activities were determined according to the procedures described in the *Worthington Enzyme Manual* (1988). Protein concentrations were determined with a Bio-Rad reagent (Bradford, 1976).

Peroxidase and tyrosinase standards were also purchased from Sigma. About 100 units of tyrosinase was incubated with various concentrations of artemisinin, 2,4-D, or glyphosate for 60 min at room temperature. Similarly, the direct effects of these chemicals on various units of peroxidase in 1 mL were also determined. At the end of the incubation period, 3–4 mL of substrate, with or without dye, in buffer solution was added to each tube. Initial optical density (OD) was immediately determined for 10 min for tyrosinase and for 2 min for peroxidase.

RESULTS

Artemisinin and glyphosate at 5, 10, and 20 μM inhibited root induction in mung bean (Table I). At 5 μM 2,4-D some roots were visible, indicating a threshold level for this compound. The fresh weight of the seedlings treated with artemisinin was 19–26% less than control; glyphosate-treated seedling fresh weight was inhibited 16 and 19% at 10 and 20 μM, respectively. On the other hand, 2,4-D stimulated fresh weight of the seedlings by 10–35% (Table

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Table I. Effects of Artemisinin, 2,4-D, and Glyphosate on Root Induction and Growth of Mung Bean Seedling Cuttings

treatment	concn, μM	root no.	weight, mg fresh	weight, mg dry
control	0	8.09 ± 1.92	100.10 ± 1.38	9.70 ± 1.72
artemisinin	5	0.00 ± 0.00	81.53 ± 3.02	6.09 ± 0.51
	10	0.00 ± 0.00	73.71 ± 8.53	5.34 ± 0.66
	20	0.00 ± 0.00	75.74 ± 4.79	4.60 ± 0.70
2,4-D	5	0.84 ± 0.84	135.85 ± 1.57	9.69 ± 1.57
	10	0.00 ± 0.00	116.34 ± 4.28	8.01 ± 0.83
	20	0.00 ± 0.00	110.47 ± 2.56	7.53 ± 1.59
glyphosate	5	0.00 ± 0.00	101.80 ± 2.18	8.56 ± 0.29
	10	0.00 ± 0.00	84.65 ± 3.38	7.75 ± 1.20
	20	0.00 ± 0.00	81.55 ± 7.72	7.50 ± 1.95

I). Dry weight of the seedlings was inhibited 37, 45, and 53% by artemisinin at 5, 10, and 20 μM, respectively. Glyphosate inhibited dry weight by about 12, 20, and 23% at the concentrations tested, while 2,4-D-treated seedlings decreased 0, 17, and 22% of the control (Table I).

The time required for artemisinin and glyphosate to affect root induction in mung bean seedlings was determined. As shown in Table II, only 2 days was required to inhibit root formation of the seedlings treated with 10 μM artemisinin. Glyphosate at 10 μM required more than 4 days to completely inhibit root induction. However, most of the roots even after 1 day were less than 0.2 cm long for both compounds (Table II).

Since artemisinin, glyphosate, and 2,4-D all inhibited root formation in mung bean, we questioned whether the effects were similar at the biochemical level. Because peroxidase and tyrosinase are important enzymes in phenolic metabolism and lignin biosynthesis and these processes are essential to the formation of new vascular systems in root growth, we tested their synthesis and activity in mung bean seedlings treated with 10 μM artemisinin, glyphosate, and 2,4-D. As shown in Table III, artemisinin significantly inhibited the endogenous peroxidase content in the treated stem tissue. Glyphosate-treated tissue was not different from the control, while 2,4-D increased the endogenous peroxidase content 3-fold.

Protein, peroxidase, and tyrosinase in the medium following 7 days of mung beam culture with artemisinin, glyphosate, or 2,4-D were determined. After the volume of the medium was adjusted to the beginning amount (10 mL), duplicate 1-mL samples were removed for assay. There were no differences among the treatments for total protein in the spent medium, and tyrosinase was not found at the minimum level of detection (data not shown). Peroxidase was increased over control in 2,4-D-treated spent medium (Figure 1). In artemisinin-treated spent medium, very little peroxidase was detected. Glyphosate had no effect on the secretion of peroxidase.

Artemisinin had no effect on the activity of tyrosinase obtained from a commercial source even when incubated with 100 μg/mL artemisinin (Table IV). Glyphosate and 2,4-D were both inhibitory at concentrations of 10 μg/mL and above.

DISCUSSION

The importance of natural herbicides has been emphasized by a number of symposia held by the American Chemical Society (Cutler, 1988) and by the Weed Science Society of America (Putnam, 1988). Duke and Lydon (1987) have pointed out that the success of natural compounds as commercial herbicides will depend on several factors: the policy of regulatory agencies toward toxicological screening and licensing, the cost of production, the efficacy and selectivity of the compounds in the field, and the success of industry in patenting. Two groups of scientists, led by Duke (1987, 1988) and Chen (1987, 1990), independently, showed that artemisinin has potential as a natural herbicide. Artemisinin is a drug that is available in China. Its toxicity has been extensively studied in humans and other animals and found to be absent (Klayman, 1985; Luo and Shen, 1987). The selectivity of artemisinin (Duke et al., 1987) and its potency (Chen and Leather, 1990) would be additional advantages to consider artemisinin as a potential herbicide.

The question may be raised as to whether a natural product generally is not as effective as synthetic herbicides. In this study, we chose two commonly used herbicides, 2,4-D and glyphosate, for comparative studies in mung bean seedling cutting bioassay systems. We considered parameters such as root induction, growth (both in fresh and dry weights), and the time required for more than 50% inhibition of root induction in the treated mung bean seedling cuttings. Our results indicate that artemisinin may not be as effective as 2,4-D; however, they suggest that artemisinin is much more potent than glyphosate.

The mode of action of artemisinin in malaria has been reviewed (Luo and Shen, 1987). In relation to cell structure, artemisinin interferes with membrane of the parasite. The treated parasite showed distended mitochondria, swollen outer mitochondrial and nuclear membranes, dissociation of ribosomes with endoplasmic vacuolization, and, finally, formation of autophagic vacuoles which caused degeneration and eventual death. In relation to biochemistry, artemisinin interfered with the rate of lactate production and did not markedly inhibit carbohydrate metabolism of *Plasmodium falciparum*. At 5–50 μM artemisinin and its derivatives inhibited the uptake of [^{3}H]isoleucine. This suggests that protein synthesis may be the primary target of attack. However, the mode of action of artemisinin in plants is not yet known.

Because glyphosate affects the aromatic amino acids and shikimic metabolism (Becerril et al., 1989; Cole, 1985; Jaworski et al., 1984) and because peroxidase is widely distributed in the plant kingdom and also occurs in the animal kingdom (Gaspar et al., 1982; van Huystee, 1989), tyrosinase and peroxidase were selected for possible influence by artemisinin, 2,4-D, and glyphosate.

Our results showed that glyphosate and 2,4-D are inhibitors of tyrosinase. Artemisinin, unlike 2,4-D and glyphosate, did not show significant effect on tyrosinase activity. This suggests that the mechanism of action of artemisinin is different from that of glyphosate and 2,4-D.

Interestingly, all these tested chemicals showed significant inhibitory effects on root induction. Apparently, the mechanism of action of these compounds was quite different. Artemisinin showed inhibitory effects on endogenous peroxidase. On the other hand, 2,4-D drastically increased peroxidase activity in stem basal tissue.

When peroxidase activity was determined from the spent media, our results consistently showed that (a) spent medium containing artemisinin had very little peroxidase activity and (b) 2,4-D stimulated peroxidase synthesis in the tissue and its release into the spent medium. However, when artemisinin and other chemicals were separately added to various concentrated peroxidase preparations, no significant direct effect on this enzyme's activity was observed. The results shown in Table III and Figure 1 seem to suggest that artemisinin actually inhibits peroxidase synthesis in the treated tissue.

Table II. Effect of Treatment Time (Days) on Root Inhibition by Artemisinin and Glyphosate at 10 μM[a]

	artemisinin				glyphosate			
time, days	total	long	medium	short	total	long	medium	short
0	8.3 ± 2.2	7.1 ± 2.8	0.8 ± 1.8	0.3 ± 0.7				
1	7.8 ± 7.7	1.0 ± 1.4	2.0 ± 4.4	4.8 ± 3.6	7.7 ± 1.8	0.2 ± 0.3	0.5 ± 1.1	6.5 ± 3.2
2	0.3 ● 0.7	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.7	7.8 ± 6.6	0.2 ± 0.4	0.2 ± 0.4	7.4 ± 6.8
4	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.0	6.6 ± 9.4	0.0 ± 0.0	0.0 ± 0.0	6.6 ± 9.4
7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Length of roots: long = >1 cm, medium = 0.2–0.9 cm, short = <0.2 cm.

Table III. Endogenous Peroxidase in Stem Tissue Treated with Artemisinin, 2,4-D, or Glyphosate (10 μM)

treatment	total peroxidase, units	protein content, mg/g	specific activity, units/g
control	25	1.95	12.82
artemisinin	11	3.20	3.44
2,4-D	85	2.25	37.78
glyphosate	47	3.55	13.24

Table IV. Direct Effect of Artemisinin, 2,4-D, and Glyphosate on Tyrosinase Activity[a]

	tyrosinase activity (OD) after treatment		
concn, μg/mL	artemisinin	2,4-D	glyphosate
0	0.327 ± 0.004	0.344 ± 0.005	0.360 ± 0.001
10	NT	0.163 ± 0.002	0.072 ± 0.002
20	0.314 ± 0.003	0.143 ± 0.000	0.056 ± 0.001
50	0.345 ± 0.004	0.050 ± 0.000	0.056 ± 0.001
100	0.316 ± 0.008	0.055 ± 0.001	0.047 ± 0.001

[a] Tyrosinase = 100 units/mL; incubation time = 60 min at 25 °C; NT = not tested due to no significant difference at higher concentrations tested. All the experiments were repeated twice with three replicates for each time. $p = 0.005$.

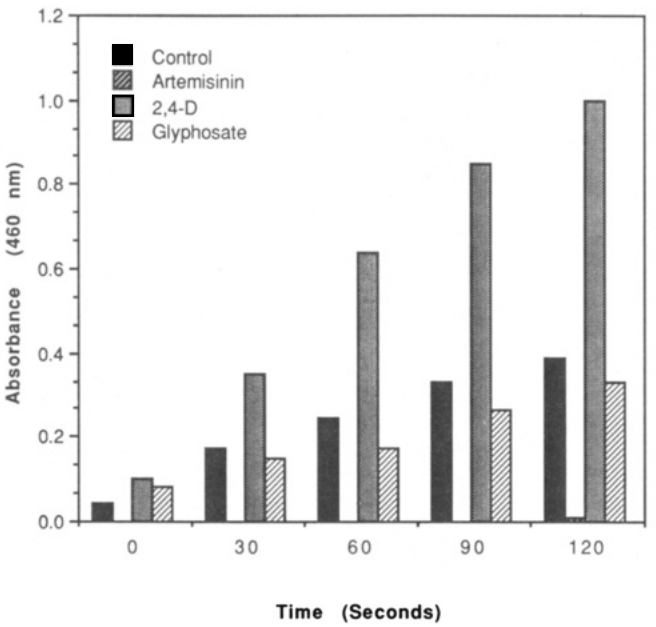

Figure 1. Total peroxidase activity in the spent media after mung bean seedling cuttings were incubated in the media containing various compounds for 1 week in a walk-in growth chamber.

When all the parameters, such as root induction and fresh and dry weights of the treated basal tissues, were considered, artemisinin, although not as good as 2,4-D in some parameters, was definitely more effective than glyphosate in a number of parameters compared (Tables I and II). Thus, our results further support the desirability of considering artemisinin as a potential natural herbicide in field studies.

Furthermore, 2,4-D has been in use since 1942 and glyphosate since 1972. It is well-known in agriculture and medicine that prolonged use of any chemical may lead to tragic events. Artemisinin has been proven to be nontoxic, and no known toxic metabolites have been found from animal and human studies (Klayman, 1985; Luo and Shen, 1987). Artemisinin is a sesquiterpene made of carbon, hydrogen, and oxygen and lacks the chlorine found in 2,4-D and the nitrogen and phosphate found in glyphosate. Thus, artemisinin deserves more attention for its potential use in agriculture.

The main problem in using artemisinin as a herbicide is its availability. Artemisinin is a secondary metabolite of *Artemisia annua*, and its content in the naturally grown plants is very low (Klayman, 1985). Although reports have shown that artemisinin can be chemically synthesized [see Luo and Shen (1987)], the chemical synthesis involves many steps and some unusual conditions that made the process very expensive. Thus, alternative sources of artemisinin should be considered: (a) plant breeding or genetic manipulation of plants to improve artemisinin content, (b) chemical spraying before harvesting of the plant tissue to increase artemisinin, or (c) plant tissue culture biotechnology to produce artemisinin through fermentation or biotransformation of other related chemicals to artemisinin in bioreactors.

Among these three possible alternatives, the third one is perhaps the most studied. Some research work has been carried out by a number of investigators. Nair et al. (1986) described the production of artemisinin in callus and root organ cultures derived from *A. annua*. Kudakasseril et al. (1987) investigated the effect of sterol inhibitors on the incorporation of [^{14}C]isopentenyl pyrophosphate into artemisinin by a cell-free system from *A. annua* tissue cultures and plants. Chen et al. (1988) reported the biotransformation of artemisic acid, arteannuin B, and some other natural sesquiterpenes to artemisinin by cell culture of *A. annua*. Recently, Kreis and Reinhard (1989) have reviewed the production of secondary metabolites by plant cells cultivated in bioreactors. They pointed out that plant cell cultures are a potential source of pharmaceutically important plant metabolites. They estimated that the production of 20 000 L may take less than 10 weeks, which is a more shorter time than that required to grow the plants in the field. The cost analysis available shows that the production of valuable chemicals by a suitable plant cell culture process would be commercially viable.

Artemisinin is an antimalarial agent that has been used in tropical medicine because it is more potent than quinine derivatives. The paucity of cases of malaria in the United States results in little interest in the use of artemisinin in that context. However, as our agricultural industry probably is the largest consumer of pesticides, a natural herbicide should be of great interest. Artemisinin is not only important in tropical medicine but may play an important role in our agriculture. For these two reasons, artemisinin can be considered a valuable natural product

for medicine and agriculture. Further field studies, attempts to increase production in plants and by tissue culture, and biotechnological research on this potential natural herbicide are warranted.

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