

The Influence of Formulation, Buffering, pH and Divalent Cations on the Activity of Endothall on Hydrilla¹

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ABSTRACT

Endothall has been used as an aquatic herbicide for more than 40 years and provides very effective weed control of many weeds. Early research regarding the mechanism-of-action of endothall contradicts the symptomology normally associated with the product. Recent studies suggest endothall is a respiratory toxin but the mechanism-of-action remains unknown. To further elucidate the activity of endothall, several endothall formulations were evaluated for their effects on ion leakage, oxygen consumption and photosynthetic oxygen evolution from hydrilla shoot tips. The influence of pH, buffering and divalent cations was also evaluated. Based on ion leakage, the LD₅₀ of the alkylamine formulation was substantially lower under both light and dark conditions than the acid or dipotassium salt formulations. Lowering the pH increased ion leakage, and therefore activity, of the dipotassium salt, while buffering had the opposite effect on the acid formulation. Neither pH nor buffer concentration had an effect on the alkylamine salt, however addition of divalent cations increased ion leakage by all formulations. The rate of oxygen consumption was initially increased followed by a sharp decrease for both the dipotassium and alkylamine salt formulations. All formulations also caused a marked reduction in photosynthetic oxygen evolution within 60 minutes of treatment. Based on these data, endothall appears to inhibit respiration and photosynthesis, possibly due to a similar mechanism. There also appears to be fundamental differences between the alkylamine salt and the other formulations that may help to explain the mechanism-of-action of endothall in hydrilla and other plants.

Key words: mode-of-action, conductivity, photosynthesis inhibition, oxygen consumption, uncoupler, ionophore.

INTRODUCTION

Endothall (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid) has been widely used for effective control of several submersed aquatic species including hydrilla (*Hydrilla verticillata* [L.f.] Royle), Illinois pondweed (*Potamogeton illinoensis* Morong), and southern naiad (*Najas guadalupensis* [Spreng.] Magnus) (Hiltibran 1963, Thayer et al. 2001). Endothall is also registered for use as a preharvest defoliant for cotton (*Gossypium hirsutum* L.), as a potato vine (*Solanum tuberosum* L.) desiccant and harvest aid for legume forages (Anonymous 2002).

Endothall was first discovered in 1929 by vonBruchhausen and Bersch as a derivative of cantharidin, a natural product of the blister beetle (*Epicauta* spp. - Davidson and Lyon 1979) and was reported to possess herbicidal activity in 1948 by Niagara Chemical Company. This compound was registered for use as a plant growth regulator by the Sharples Chemicals Company in 1951 (Tischler et al. 1950). Endothall was first registered for aquatic weed control in 1960 by Pennsalt Chemical Company (Hiltibran 1963).

Several formulations of endothall have been developed and marketed and include the sodium salt which is no longer registered for use, the dipotassium salt, and mono- and dimethylalkylamine salt formulations. The sodium and dipotassium formulations form salts with the parent acid at the carboxylic acid moieties. This is similar to the alkylamine, which either forms salts at one carboxylic acid group (mono-alkylamine) or both (di-methylalkylamine). The alkyl groups are a mixture of C₁₀ to C₁₂ straight chains, although chain length varies from 8 to 14 carbons (Anonymous, 1976).

Both dipotassium (Aquathol® K) and alkylamine (Hydrothol® 191) salts of endothall are formulated as liquid or granule and are registered for aquatic use. The dipotassium salt is recommended for hydrilla control at 0.5 to 3.0 ppm and the alkylamine from 0.05 to 0.5 ppm. While the alkylamine salt formulation is more effective, its use is limited in several areas due to fish toxicity issues (Thayer et al. 2001). The oral LD₅₀ (rat) for endothall is also variable with respect to formulation. The LD₅₀ for the parent acid is 38 to 51 mg/kg while the dipotassium and alkylamine salts are 198 and 206 mg/kg, respectively (Ahrens 1994). This discrepancy between lower LD₅₀, yet higher fish toxicity for the alkylamine salt is not well understood.

Endothall dissipates very rapidly after application. The half-life of the dipotassium salt is often as short as 2 to 3 days in water while alkylamine salts may persist for 14 to 21 days

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⁷50mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-NaOH, pH 7.

Model 820, Orion Research, Boston, MA.

(Simsimann et al. 1975). Microbial breakdown is the primary means of dissipation in the aquatic environment (Sikka and Saxena 1973). Endothall has limited soil activity in terrestrial systems and is taken up by the roots and translocated apoplastically (Turgeon et al. 1972). There is no appreciable phloem movement and post-emergence selectivity among terrestrial plant species appears to be differential absorption, uptake, or by both processes. In contrast, movement within aquatic plants is symplastic (Thomas and Seaman 1968).

Endothall appears to be contact in activity, but research on its mode-of-action indicates otherwise. Mann and Pu (1968) showed that endothall inhibited lipid synthesis and caused a decrease in protein synthesis (Mann et al. 1965). Tsay and Ashton (1971) showed a reduction in dipeptidase and proteinase activity, while Penner and Ashton (1968) showed this decrease was similar to actinomycin D and therefore postulated that endothall interfered with mRNA metabolism.

Collectively, the modes-of-action proposed in the preceding paragraph describe a slow-acting herbicide, quite in contrast to the rapid, contact-type activity of endothall noted in field studies. Research by MacDonald et al. (1993, 1999) has reported that endothall may have similar activity in plants and animals, and indicated respiratory toxicity. In an attempt to further elucidate the mechanism-of-action of endothall, several studies were conducted to evaluate the influence of formulation, buffering, pH, and divalent cations on endothall activity. Hydrilla was used as an assay system due to its susceptibility to endothall and the importance of this weedy aquatic species (Langeland 1990).

MATERIALS AND METHODS

Preparation of hydrilla shoots. Plant material for all experiments consisted of apical shoots 2 cm in length collected from dioecious hydrilla cultured under greenhouse conditions. Each shoot tip contained 20 to 30 leaves, with each individual leaf approximately 2 to 3 mm wide by 7 to 10 mm long. The mean fresh weight of a blotted dry sample was 200 ± 30 mg (standard deviation). Shoot tips were excised with dissecting scissors, washed with tap water and stored in sterile water for at least one hour prior to use in experiments. Based on preliminary experiments with deionized water and microscopic observations (data not shown), this treatment maximized cell turgor and allowed the formation of callose at the excision site. Technical grade endothall formulations of the alkylamine salt, dipotassium salt, and acid monohydrate were used for all studies. All herbicide formulations were supplied by Cerexagri, Inc.

Ion Leakage Studies. Hydrilla shoot tips (one tip per 5 dram vial) were incubated in 5 ml of treatment solution, similar to previous studies (MacDonald et al. 1993). Identical treatments were either in continuous light or in constant darkness for the duration of studies. Treatments maintained in continuous light ($300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were held in clear 5 dram vials in a water bath shaker at 21 C. Dark treatments were maintained in vials on a rotary shaker in a photographic darkroom at 26 C. Conductivity ($\mu\text{mhos}/\text{cm}^2$) was measured utilizing a conductivity bridge⁶ with the probe modified to contain the entire 5 ml of treatment solution. Initial conductivity was measured on the solutions alone and total conduc-

tivity was obtained by freezing and thawing the samples twice to release all ions. Data are presented as percent conductivity derived from the following equation: % conductivity = $((\text{measured} - \text{initial}) / (\text{total} - \text{initial})) * 100$; where measured equaled the amount of conductivity at each time of measurement.

For initial comparisons of formulation, ion leakage from hydrilla shoot tips was monitored in continuous light or complete darkness at 4, 8, 12, 24, 48 and 72 hours after initial treatment. A concentration of 100 μM was used for all formulations with deionized water as a control. A dose response over a 24-hour period was obtained for all formulations and this was used to calculate the I_{50} (the concentration required to cause 50% ion leakage) for continuous light and constant dark treatments. To evaluate the effect of pH, 50 μM alkylamine and 2 mM dipotassium salt formulations were placed in unbuffered solutions at pH 3, 5, or 7. Hydrochloric acid (HCl) or potassium hydroxide (KOH) was used to achieve desired pH. The effect of buffering⁷ and ammonium chloride (2 mM) was also tested on the acid and alkylamine formulations of endothall. In addition, 2 mM concentrations of monovalent and divalent cations were tested for their effects with all endothall formulations. Ion leakage was monitored for 24 hours for pH, buffering and cation studies. Percent ion leakage data was adjusted for the amount of leakage that occurred in control treatments. All experiments were conducted at least twice with a minimum of four replications.

Oxygen Uptake. Hydrilla shoot tips were placed in treatment solutions containing 10 or 100 μM of alkylamine salt or dipotassium salt formulation of endothall. Distilled, deionized water was used as a control. Dark respiration was monitored by first acclimating shoot tips in complete darkness for 1.0 hour then placing them in 15ml of treatment solution in a 5 dram glass vial, maintaining complete darkness. A dissolved oxygen probe⁸ was wrapped with teflon tape so that it was sealed in the mouth of the vial just as head-space (air) was eliminated. Agitation was provided by clamping the vial in a wrist-action shaker. Oxygen uptake as a measure of respiration was measured over 10-minute periods at hourly intervals for 4 hours. Reported results are the means of eight replications.

Photosynthetic Oxygen Evolution. Photosynthesis was assayed as light-induced oxygen evolution from hydrilla shoot tips in 15 ml of treatment solution. Treatments included distilled water control, 2,000 μM acid monohydrate, 2000 μM dipotassium salt or 100 μM alkylamine salt of endothall. Shoot tips were placed in a 5 dram glass vial containing the treatment solution and 5 mM sodium bicarbonate (NaHCO_3). The vial was clamped in a wrist-action shaker and exposed to $300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. Tips were positioned so that light could be focused on the abaxial and adaxial surfaces of the leaves. The oxygen meter probe⁸ was wrapped with teflon tape and placed in the vial so that it sealed just as head-space was eliminated. Measurements were recorded for 1 to 5 minutes, depending on the rate, hourly for 2 hours. Between measurements the samples were stored in an illuminated water bath shaker. The reported results are the means of 6 replications.

Statistics. Data were subjected to analysis of variance (ANOVA) to determine the significance of treatment effects

and interactions ($P < 0.05$). Data for ion leakage were pooled across experiments and means separated using Fisher's least significant difference procedure at the 0.05 level. Means on the effect of formulation on oxygen consumption and photosynthetic oxygen evolution were separated using Fisher's least significant procedure at the 0.05 level. Results for other studies are presented with standard errors of the mean with a minimum of four replications.

RESULTS

Ion Leakage. Initial conductivity experiments showed that the alkylamine formulation of endothall caused greater damage in both light and dark than either of the other formulations. The alkylamine salt caused over 60% conductivity within 4 hours of treatment and almost complete ion leakage after 72 hours in the light (Figure 1). There was clearly a greater effect of this formulation on ion leakage in darkness, where $> 95\%$ conductivity was measured after 4 hours and complete ion leakage had occurred with 24 hours of treatment (Figure 2). The dipotassium salt and acid formulations showed $< 25\%$ conductivity in the light with little difference between formulation over the treatment period (Figure 2). In the dark, endothall acid caused more rapid and greater ion leakage as compared to the dipotassium salt formulation (Figure 2). Based on I_{50} values, the alkylamine formulation was nearly 100 times more toxic than either the acid or dipotassium salt formulations (Table 1). There was no difference in I_{50} between the acid and dipotassium salt in the dark.

Changes in pH had no effect on the activity of the alkylamine salt (Table 2). Acidification of the medium greatly enhanced the activity of dipotassium endothall and this effect was observed under both light and dark conditions. There was no impact of buffering, the addition of ammonium chloride or the combination of the two on ion leakage from the alkylamine formulation in the light (Table 3). Alkylamine activity was enhanced by buffering alone in the dark and the activity of buffering was greater with ammonium chloride.

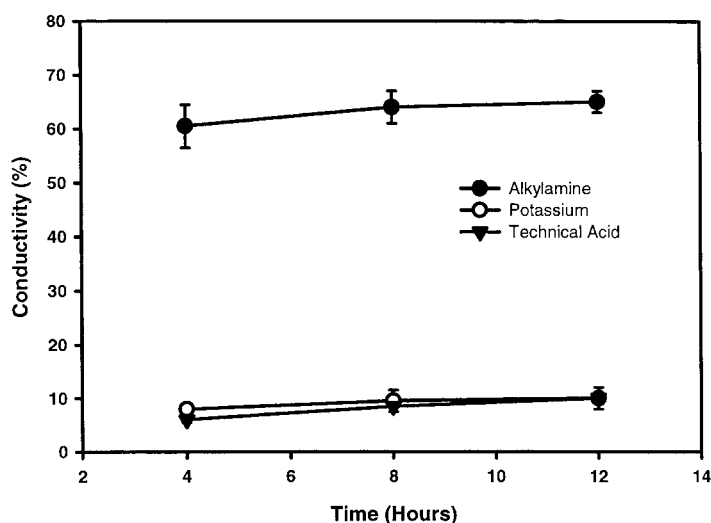


Figure 1. The effect of 100 μM endothall technical acid, dipotassium salt, and alkylamine salt on ion leakage from hydrilla shoot tips in the light.

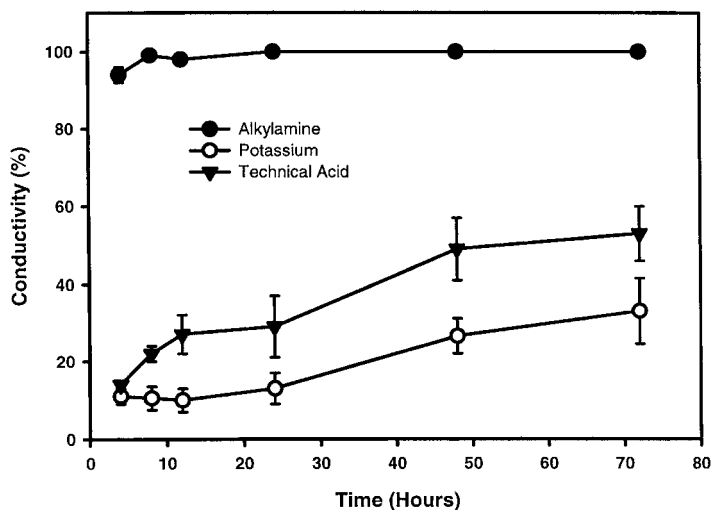


Figure 2. The effect of 100 μM endothall technical acid, dipotassium salt, and alkylamine salt on ion leakage from hydrilla shoot tips in the dark.

Conversely, buffering the treatment medium greatly decreased the activity of technical endothall acid under both light and dark conditions. The addition of ammonium chloride to buffer ameliorated this decrease in activity in the light but had little impact under dark conditions. The addition of ammonium sulfate and the divalent cations iron, calcium or magnesium caused a dramatic increase in activity across all formulations of endothall (Table 4). The dipotassium salt formulation showed slightly less activity compared to the other formulations and there was little difference between treatments under light or dark conditions.

Oxygen Uptake. Both dipotassium and alkylamine salts of endothall, regardless of concentration tested, caused an increase in oxygen consumption immediately after treatment (Table 5). The alkylamine salt continued to increase oxygen consumption after one hour while the dipotassium salt decreased the level of oxygen being utilized. After two hours of treatment, both concentrations of the dipotassium salt and 100 μM alkylamine significantly reduced oxygen consumption.

Photosynthetic Oxygen Evolution. All formulations of endothall inhibited photosynthetic oxygen evolution at the doses tested, and caused complete inhibition within 2 hours (Table 6). There was no effect of the acid formulation after 10 minutes, but the dipotassium and alkylamine formulations of endothall inhibited photosynthesis within 10 minutes by 72 and 82%, respectively. Endothall alkylamine caused complete inhibition after 1 hour.

TABLE 1. I_{50} VALUES FOR THREE ENDOTHALL FORMULATIONS BASED ON ION LEAKAGE OVER 24 HOURS.

Formulation	I_{50} in light	I_{50} in dark
acid monohydrate	2.68 mM	2.71 mM
dipotassium salt	3.91 mM	2.83 mM
alkylamine salt	0.09 mM	0.04 mM

TABLE 2. THE EFFECT OF PH ON ION LEAKAGE (% CONDUCTIVITY) FROM HYDRILLA SHOOT TIPS IN THE LIGHT AND THE DARK FOR TWO SALT FORMULATIONS OF ENDOTHALL.

Formulation	Light			Dark		
	pH					
	3	5	7	3	5	7
dipotassium (2 mM)	80 a ¹ ± 6 ²	43 b ± 2	26 c ± 4	81 a ± 2	61 b ± 3	49 c ± 4
alkylamine (50 µM)	36 a ± 2	49 a ± 2	31 a ± 6	79 a ± 3	79 a ± 2	67 a ± 8

¹Means within formulation and light regime followed by a different letter are significantly different ($\alpha = 0.05$, Fisher's least significant difference procedure).

²Means followed by standard error.

DISCUSSION

The alkylamine formulation of endothall was found to be much more active than either the acid or dipotassium (K⁺) salt, causing greater and more rapid ion leakage in hydrilla. This effect was similar under light or dark conditions, with greater ion leakage under darkness. MacDonald et al. 1993 also found greater leakage under dark conditions for the parent acid, similar to the results observed in this study. However, the most striking observation was the concentration difference between the alkylamine and the other formulations. The alkylamine exhibited activity at rates 97% and 98% lower concentrations than the acid and K⁺ salt, respectively. Walker (1963) also reported major differences between the disodium salt and the alkylamine salt of endothall with respect to herbicidal activity. He reported a nearly 100-fold greater activity in fish mortality of fish exposed to the alkylamine salt compared to the disodium salt which is similar to the nearly 100-fold difference in activities reported here. This is also similar to the use rates for aquatic weed control under field conditions. Typical use rates for the alkylamine salt range from 0.1 to 3.0 ppm, while rates for the dipotassium salt range from 1.0 to 5.0 ppm (Thayer et al. 2001).

Decreasing pH and the addition of ammonium chloride had little impact on the activity of the alkylamine, but

dramatically increased the activity of the K⁺ salt and acid formulations. These data suggest that the more undissociated the acid and K⁺ salts become the greater activity, presumably due to increased uptake of a more neutral molecule. This has been reported for many weak acid herbicides such as 2,4-D (2,4-dichlorophenoxy) acetic acid, where permeability through the cell membrane increases with decreasing pH (Briggs et al. 1987, Devine et al. 1987). The alkylamine salt appears to react differently, suggesting that this formulation does not dissociate from the parent acid, at least in the conditions present in this study. It also suggests that the alkylamine formulation is more lipophilic than either the acid or K⁺ salt and may be forming a micelle (Benjamin Horenstein, pers. comm.). Another possibility could be that the alkylamine salt dissociates from the parent acid and reacts directly with the membrane to increase permeability for the parent acid to be absorbed.

Buffering dramatically reduced the activity of endothall acid under both light and dark but had little effect on the alkylamine. Buffering the solution at pH 7.0 maintains endothall acid in the dissociated form, limiting uptake. The addition of a proton donor such as ammonium chloride enhanced activity, allowing more of the endothall acid to be undissociated.

TABLE 3. THE EFFECT OF BUFFERING AND AMMONIUM CHLORIDE ON ION LEAKAGE (% CONDUCTIVITY) FROM HYDRILLA SHOOT TIPS IN THE LIGHT AND THE DARK FOR TWO FORMULATIONS OF ENDOTHALL.

Treatment	Light		Dark	
	Amine ¹	Acid	Amine	Acid
none	75 ± 8 ²	53 ± 4	53 ± 9	52 ± 6
buffer ³	82 ± 9	25 ± 2	71 ± 2	20 ± 2
NH ₄ Cl ⁴	79 ± 12	79 ± 3	49 ± 9	59 ± 6
buffer + NH ₄ Cl	88 ± 8	59 ± 3	84 ± 9	22 ± 9
LSD _{0.05} ⁵	NS	12	19	17

¹Concentrations for amine and acid formulations are 50 µM and 2 mM, respectively.

²Means followed by standard error.

³50mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) - NaOH, pH 7.

⁴2 mM ammonium chloride.

⁵Mean comparison within a column ($\alpha = 0.05$, Fisher's least significant difference procedure).

TABLE 4. THE EFFECT OF AMMONIUM AND DIVALENT CATIONS ON ION LEAKAGE (% CONDUCTIVITY) FROM HYDRILLA SHOOT TIPS IN THE LIGHT AND THE DARK FOR THREE FORMULATIONS OF ENDOTHALL.

Treatment ¹	Light			Dark		
	Amine ²	AK ⁺ salt	Acid	Amine	AK ⁺ salt	Acid
none (D.I. Water)	28 ± 4 ³	27 ± 3	53 ± 7	59 ± 10	49 ± 4	50 ± 6
NH ₄ (SO ₄) ₂	75 ± 5	—	81 ± 3	91 ± 8	—	85 ± 3
FeSO ₄	80 ± 2	76 ± 3	99 ± 1	82 ± 4	86 ± 4	90 ± 3
MgCl ₂	93 ± 4	74 ± 4	96 ± 3	89 ± 4	80 ± 3	88 ± 6
CaCl ₂	93 ± 6	75 ± 2	93 ± 5	93 ± 5	80 ± 4	85 ± 8
LSD _{0.05} ⁴	9	6	11	19	10	10

¹Concentrations for all treatments was 2 mM. Treatments included ammonium sulfate [(NH₄(SO₄)₂], ferrous sulfate [FeSO₄], magnesium chloride [MgCl₂], and calcium chloride [CaCl₂].

²Concentrations for amine (alkylamine), K⁺ (potassium) salt and acid formulations were 50 µM, 2 mM and 2 mM, respectively.

³Means followed by standard error.

⁴Mean comparison within a column ($\alpha = 0.05$, Fisher's least significant difference procedure).

TABLE 5. THE EFFECT OF TWO FORMULATIONS OF ENDOTHALL ON DARK RESPIRATION (OXYGEN CONSUMPTION) OF HYDRILLA SHOOT TIPS.

Treatment	Concentration	Hours after initial exposure				
		0	1	2	3	4
		----- oxygen consumption (nmol-g ⁻¹ min ⁻¹) -----				
distilled water	—	90 ± 15 ¹	85 ± 0	58 ± 0	50 ± 0.4	1 ± 0
potassium salt	10 µM	143 ± 15	58 ± 10	12 ± 5	0 ± 0	0 ± 0
potassium salt	100 µM	215 ± 38	30 ± 12	0 ± 0	0 ± 0	0 ± 0
alkylamine salt	10 µM	170 ± 0	343 ± 38	58 ± 17	0 ± 0	0 ± 0
alkylamine salt 100 &M		135 ± 30	340 ± 82	10 ± 3	0 ± 0	0 ± 0
LSD _{0.05} ²		79	135	26	1.2	79

¹Means followed by standard error.

²Mean comparison within a column ($\alpha = 0.05$, Fisher's least significant difference procedure).

Divalent cations greatly enhanced the activity of all formulations, with slightly less enhancement observed for the K⁺ salt. This could be due to competition between the cations and the K⁺ itself. The cations could also be reacting directly to form a different salt (molecule). Chelation between the mono-oxygen bridge of endothall and divalent cations such as Mg have been reported (Matsuzawa et al. 1987). This chelation could act to stabilize the molecule, making it more lipophilic and consequently increasing diffusion through membranes. Acidification of the media could also have occurred by hydrilla absorbing the cations, exchanging protons for cation uptake.

The effect of the alkylamine and K⁺ salt formulations on dark respiration was similar, with both causing an initial stimulation of respiration followed by complete cessation of oxygen consumption within 3 hours. However, the alkylamine salt caused a longer stimulation of respiration compared to the K⁺ salt. This stimulation effect was previously reported with the acid formulation by MacDonald et al. (1993). If we are to assume that the greater activity of the alkylamine salt is due to more rapid and complete absorption, then one would have expected the alkylamine salt to show greater impact on respiration compared to the K⁺ salt. However, the K⁺ salt showed a more rapid decline in respiration compared to the alkylamine salt. The effect of formulation on photosynthetic oxygen evolution was similar to the trends observed for ion leakage, where the alkylamine salt severely reduced oxygen evolution after 10 minutes and complete cessation after 60 minutes.

TABLE 6. THE EFFECT OF THREE FORMULATIONS OF ENDOTHALL ON PHOTOSYNTHETIC OXYGEN EVOLUTION FROM HYDRILLA SHOOT TIPS.

Treatment	Concentration	Hours after initial exposure		
		0.16	1.0	2.0
		-- oxygen evolution (nmol-g ⁻¹ min ⁻¹) --		
distilled water	—	619 ± 125 ¹	569 ± 114	612 ± 71
endothall acid	2,000 µM	527 ± 116	232 ± 201	0 ± 0
potassium salt	2,000 µM	176 ± 48	91 ± 79	0 ± 0
alkylamine salt	100 µM	113 ± 60	0 ± 0	0 ± 0
LSD _{0.05} ²		281	426	114

¹Means followed by standard error.

²Mean comparison within a column ($\alpha = 0.05$, Fisher's least significant difference procedure).

Collectively, these studies further support the hypothesis that the activity of endothall is similar to inhibitory uncouplers such as dinoseb (2-sec-butyl-4,6-dinitrophenol) (MacDonald et al. 1993). Moreover, there were striking differences between the alkylamine formulation and the acid and K⁺ salts. The most obvious was the rate response, but also the differential impact of buffering and pH between formulations. Other interesting observations were the differences observed between the formulations and respiration and photosynthetic oxygen evolution. The alkylamine salt appeared to have greater impact on photosynthesis and less of an impact on respiration compared to the K⁺ salt.

This study does not define the primary mechanism-of-action of endothall and suggests that endothall has multiple phytotoxic modes-of-action and that the mechanism-of-action might be associated at the membrane level. The differences between formulations raise further questions regarding activity of endothall on hydrilla, suggesting that formulation, especially in the case of the alkylamine salt, may have a direct bearing on the mechanism of action of this herbicide.

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