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Title	In vitro efficacy of herbicides on Sarcocystis cruzi bradyzoites
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Citation	Japanese Journal of Veterinary Research, 69(1), 31-42
Issue Date	2021-02
DOI	https://doi.org/10.14943/jjvr.69.1.31
Doc URL	https://hdl.handle.net/2115/80618
Type	departmental bulletin paper
File Information	JJVR69-1_31-42_RieDoi.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Japanese Journal of Veterinary Research 69(1): 31-42, 2021

JJVR

REGULAR PAPER Experimental Research

In vitro efficacy of herbicides on *Sarcocystis cruzi* bradyzoites

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Received for publication, September 1, 2020; accepted, December 10, 2020

Abstract

***Sarcocystis* causes various veterinary and human infections. A few agents are available for treating *Sarcocystis* infections, but their mechanisms of action are not known. Phytohormonal herbicides are antiparasitic agents that reduce the viability of organisms belonging to the phylum, Apicomplexa. Some of these herbicides prevent Apicomplexan parasites from infecting cells and organisms, yet the effects of such drugs on *Sarcocystis* are unclear. We evaluated the activity of phytohormonal herbicides against *Sarcocystis* bradyzoites using mortality and morphological changes with methylene blue staining as an indicator. Antiparasitic activity of 17 agrochemicals that act on plant plastids—classified into the following six herbicide groups: acetyl-CoA carboxylase (ACC) inhibitors, acetolactate synthase (ALS) inhibitors, photosynthesis inhibitors, protoporphyrinogen oxidase (protox) inhibitors, carotenoid biosynthesis pathway inhibitors, very-long-chain fatty acid (VLCFA) biosynthesis inhibitors, and auxin-like herbicides—were examined. The *in vitro* system enables the screening of drugs against *Sarcocystis* species, for which a well-established culturing method is not yet available. Nine herbicides, including ALS inhibitors, ACC inhibitors, and auxin-like herbicides, caused mortality of *Sarcocystis cruzi* bradyzoites by more than 90%. Further, all ACC inhibitors caused the formation of vacuolar structures in bradyzoites, and two ALS inhibitors caused bradyzoites rounding.**

Key Words: Apicomplexa, inhibitors, *in vitro* assay, *Sarcocystis*, phytohormonal herbicides

Introduction

Sarcocystis, a coccidium in the phylum Apicomplexa, is in the family Sarcocystidae. The lifecycle of *Sarcocystis* requires an intermediate host and a definitive host that preys on the intermediate host. *Sarcocystis* species infect various animals, including mammals, birds, and reptiles[14)].

Domestic animals are intermediate hosts, and *Sarcocystis* infection leads to intestinal, muscular and cerebrospinal sarcocystosis. Infection causes death in severe cases, multiple eosinophilic myositis, and miscarriage in cattle, pigs, and sheep. The parasite causes economic losses worth millions of US dollars each year[9,13)]. Humans are definitive and intermediate hosts of some *Sarcocystis* species. *Sarcocystis* infections

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doi: 10.14943/jjvr.69.1.31

in humans as the definitive host lead to intestinal sarcocystosis, and causes mild symptoms such as transient diarrhea and stomachache. However, *Sarcocystis* infection in humans as an intermediate host causes serious symptoms, such as multiple myalgia and neurological symptoms. An outbreak of human infection with *S. nesbittii*, typically found in monkeys as intermediate hosts and snakes as definitive hosts, was reported in Malaysian in 2014[1]. Some *Sarcocystis* species also are pathogenic in many animals that are not hosts of the parasites. *Sarcocystis neurona* infects opossum as a definitive host, and cats, skunks, raccoons, and sea otters as intermediate hosts. Horses are not parasite hosts, yet *S. neurona* causes a clinical syndrome known as equine protozoal myelitis (EPM). In recent years, *S. neurona* caused fatal encephalomyelitis and increasing deaths in many marine mammals in the Northeast Pacific[5]. Further, human food poisoning cases caused by consumption of *Sarcocystis*-infected raw deer and horse meats were reported in Japan[2,3,21,30,41]. Many problems associated with *Sarcocystis* are reported from various countries, but the metabolism of *Sarcocystis* has not been completely elucidated, and therapies are limited for treatment of *Sarcocystis* infection.

Recent increased interest in evaluating effects of phytohormonal herbicides against *Plasmodium* and *Toxoplasma* is based on the close evolutionary position of the phylum Apicomplexa to algae[22,33]. Various species of Apicomplexa contain a plastid-like organelle called an apicoplast that lacks photosynthetic machinery. Further, abscisic and salicylic acids regulate the lifecycle of *Plasmodium* and *Toxoplasma*[26,29]. Thus, Apicomplexa appear to display plant-like metabolic functions. Various herbicides inhibit the growth and proliferation of *Plasmodium* and *Toxoplasma*[4,19,20,23,38,39,42]. A few reports are found in the literature for culturing specific stages of parasites of individual *Sarcocystis* species[10,12]; many other species of *Sarcocystis* cannot be cultured. A few drugs are currently used to treat equine EPM[6,11,25]. These drugs are thought to act on apicoplasts and to be effective for inhibiting proliferation of merozoites; however, details of effects and mechanisms of action are unknown.

We focused on bradyzoites, a stage of *Sarcocystis* in intermediate hosts. Bradyzoites were retained in tissues of intermediate hosts with the production of cysts by asexual reproduction until ingested by definitive hosts. However, processes and metabolism for the survival of bradyzoites are not clear. This study aimed to evaluate the effects of herbicides on *Sarcocystis cruzi* bradyzoites *in vitro* and to elucidate the underlying metabolic mechanisms.

MATERIALS AND METHODS

Collecting Sarcocystis *bradyzoites*

Heart muscles of cattle slaughtered at abattoirs were examined. Fresh cysts from 6 heart muscle specimens were identified as *Sarcocystis cruzi* morphologically: septa and bradyzoites within cysts, thin (<1 μm) cyst walls, and sparse hair-like infected with *S. cruzi* were digested with pepsin[10] to collect the *Sarcocystis* bradyzoites. Approximately 200 g of minced heart tissue was incubated with 200 mL of warm (37°C) digestive solution (2.5 g pepsin and 10 mL HCl prepared in 1,000 mL distilled water) in a water bath at 37°C for 15 min with stirring every 5 min. Homogenates were filtered through a wire mesh and filtrates were centrifuged at 900 × g for 10 min. Pellets were washed three times with phosphate buffer saline (PBS) and resuspended in Percoll solution (1:9 (v/v); 9% NaCl:Percoll). Suspensions were centrifuged at 400 × g for 10 min. Percoll solutions and host cell fractions were decanted and bradyzoite pellets were washed three times with sterile PBS using centrifugation as above. Finally, bradyzoite pellets were resuspended in sterile PBS and stored at 4°C until analysis.

Sarcocystis *mortality assay*

Viability of bradyzoites was determined using

Table 1. List of herbicides used in the current study

Herbicide name	Chemical group	Mode of action for plant	Molecular weight	Log Po /w
Clodinafop-propargyl[a)]	Allyloxyphenoxypropionic acid (fops)	Inhibition of Acetyl-CoA carboxylase (ACC)	329.43	No data
Fluazifop-butyl[b)]	Allyloxyphenoxypropionic acid (fops)		383.36	4.5
Tralkoxydim[c)]	Cyclohexanedione (dims)		349.74	4.98
Bispyribac-sodium[b)]	Pyrimidinyl benzoate	Inhibition of Acetolactate synthase (ALS)	452.35	-1.03
Orthosulfamuron[d)]	Sulfonylurea		424.44	1.31
Imazapyr[b)]	Imidazolinone		261.28	0.11
Atrazine[a)]	Triazine	Inhibition of Photosynthesis at PSII-serine 264 binders	211.26	2.61
Isouron[d)]	Urea		215.68	2.01
Pyraflufen-ethyl[e)]	Phenylpyrazole	Inhibition of Protoporphyrinogen oxidase (Protox)	261.70	3.49
Chlorphthalim[e)]	Phenylimide		413.18	2.89
Norflurazon[a)]	Pyridazinone	inhibition of Carotenoid synthesis	359.3	2.45
Isoxaflutole[a)]	Isoxazole		303.67	No data
Indanofan[a)]	Oxirane	Inhibition of Very long-chain fatty acid synthesis	340.80	3.59
2,4-dichlorophenoxyacetic acid[a)]	Phenoxyacetic	Auxin mimics	214.65	3.9
Clomeprop[b)]	Phenoxyacetic		221.04	No data
MCPP[b)]	Phenoxyacetic		324.20	No data
MCPB-ethyl[b)]	Phenoxyacetic		256.73	4.17

a): Sigma-Aldrich Co. LLC., USA, b): FUJIFILM Wako Pure Chemical Corporation, Japan, c): Tokyo Chemical Industry Co., Ltd. Japan, d): Hayashi Pure Chemical Ind., LTD., Japan, e): Nihon Nohyaku Co., Ltd, Japan,

methylene blue staining[32)]. Numbers of blue-stained dead bradyzoites per 100 total bradyzoites were counted under an optical microscope on a slide glass and expressed as a percentage of bradyzoite mortality. Average mortality rates of bradyzoites were calculated from results of three independent experiments. Statistical analysis was performed by BellCurve for Excel ver. 3.21 (Social Survey Research Information Co. Ltd., Japan). Multivariate analysis of variance and multiple comparisons with Fisher's minimum significant difference method to assess significance.

Effect of incubation temperatures, aerobic conditions, and dimethyl sulfoxide (DMSO) on bradyzoite mortality

Bradyzoites were suspended at a concentration of 10^5/mL in 5 mL of PBS. Aliquots (0.5 mL) of suspensions were stored at different incubation temperatures (37°C and 4°C) under different aerobic conditions (aerobic, microaerobic, and anaerobic). Microaerobic and anaerobic environments were generated with commercial anaerobic jars and gas packs (AnaeroPac™ Microaero and AnaeroPac™ Anaerobic, Mitsubishi Gas Chemical Company, Inc., Japan), with O_2 concentrations of 6%–12%, CO_2 concentrations 5%–8% and O_2 concentrations less than 0.1%, CO_2 concentrations of 16%, respectively. Suspensions were stored at 37°C and 4°C for 12 days and 30 days, respectively. All suspensions were observed for bradyzoites mortality every 1 to 3 days. The mortality of bradyzoite in different conditions was comparatively analyzed.

Most herbicides are poorly soluble in water. Hence, dimethyl sulfoxide (DMSO) was used as a solvent to prepare herbicide solutions. Bradyzoites suspended in PBS (10^5/mL) were incubated with different concentrations (0%, 0.1%, 0.2%, 0.5%, 0.7%, 1.0%, 2.0%, 5.0%, 7.0%, and 10% (v/v)) of DMSO at 4°C under aerobic for 10 days to determine maximum DMSO concentrations that did not affect mortality.

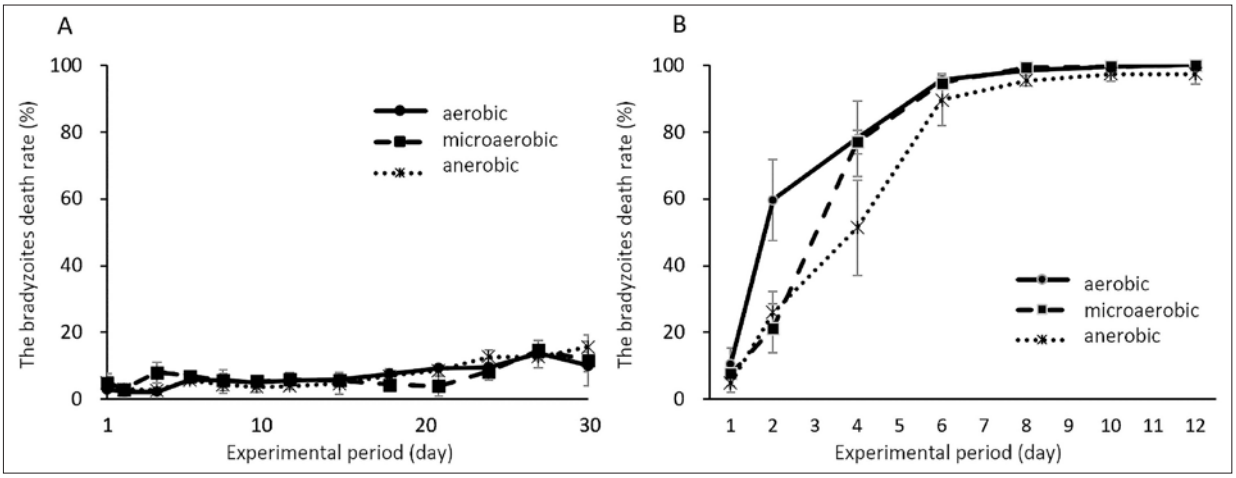


Fig. 1. Effect of different incubation temperatures and aerobic conditions on bradyzoite mortality. Error bars indicate standard deviations from three independent experiments. Bradyzoite mortality at 4°C (A) and at 37°C (B).

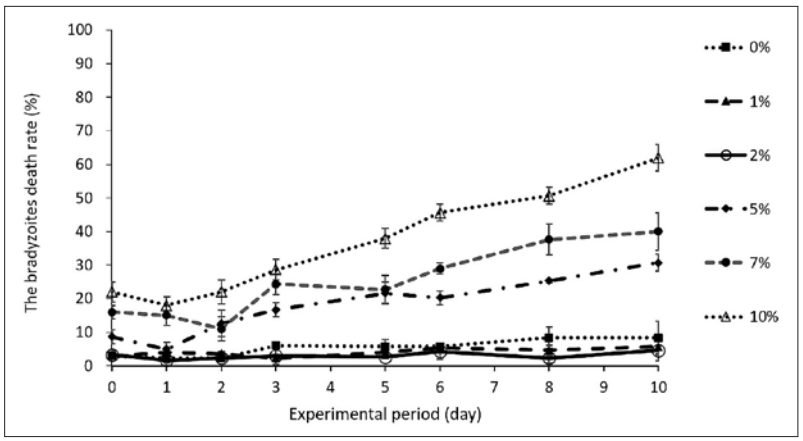


Fig. 2. Effect of different concentrations of dimethyl sulfoxide (DMSO) on bradyzoite mortality. Error bars indicate standard deviations from three independent experiments.

Herbicides

Seventeen herbicides that acted on plant plastids were selected based on mode of action[16]. The following herbicides were used (Table 1): acetyl-CoA carboxylase (ACC) inhibitors, clodinafop-propargyl, fluazifop-butyl, and tralkoxydim; acetolactate synthase (ALS) inhibitors, bispyribac-sodium, orthosulfamuron, and imazapyr; photosynthesis inhibitors, isouron and atrazine; protoporphyrinogen oxidase (protox) inhibitors, pyraflufen-ethyl and chlorphthalim; carotenoid biosynthesis pathway inhibitors, norflurazon and isoxaflutole; very-long-chain fatty acid (VLCFA) biosynthesis inhibitor, indanofan; auxin-like herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D), clomeprop, mecoprop (MCPP), and MCPB-ethyl.

Herbicide treatment

Herbicides (3 or 30 mg) were dissolved in 40 μL of DMSO and the solution was added to 2 mL of sterile PBS. Mixtures were transferred to a 24-well plate and stored at 4°C in the dark for 2 days. Next, bradyzoites—at a concentration of 10^5 /mL—were transferred to herbicide suspensions. Mortality and morphology of bradyzoites were observed immediately after transfer. Bradyzoites were incubated at 4°C in the dark and observed every 1 or 2 days for 6 days.

The herbicide suspensions that markedly

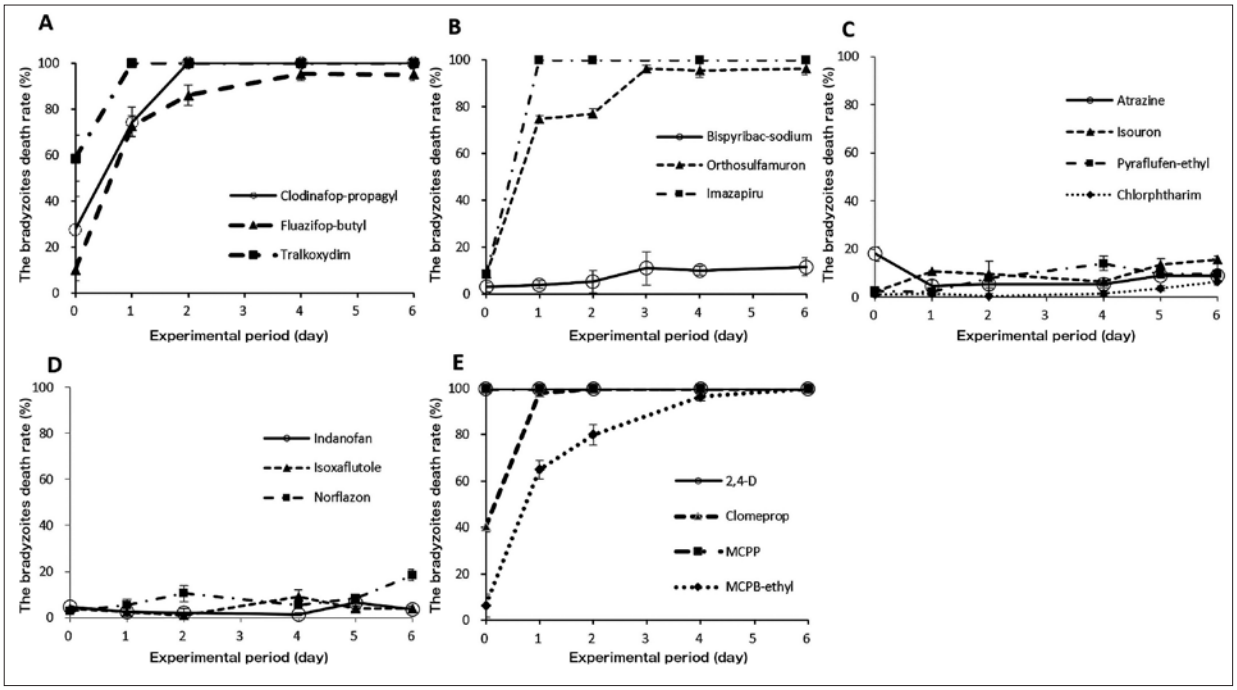


Fig. 3. Bradyzoite mortality after exposure to acetyl-CoA carboxylase (ACC) inhibitors (A), acetolactate synthase (ALS) inhibitors (B), photosynthesis and protox inhibitors (C), carotenoid synthesis inhibitors, and very-long chain fatty acid (VLCFA) synthesis inhibitors (D), and auxin-like herbicides (E). Error bars indicate standard deviations from three independent experiments.

affected viability or morphology of the bradyzoites were subjected to centrifugation at 20,000 × g for 30 min to remove the bradyzoites and undissolved drugs, as per the Guidance document of the Organization for Economic Co-operation and Development[31]. Fresh bradyzoites were added to the supernatants to confirm the effect of dissolved herbicides on mortality and morphology of bradyzoites.

RESULTS

Effect of incubation temperatures, aerobic conditions, and dimethyl sulfoxide (DMSO) on bradyzoite

Effects of different incubating temperatures and aerobic conditions on bradyzoite viability are shown in Fig. 1. Mortality of bradyzoites incubated at 37°C under varying aerobic conditions was approximately 50% on day 2 and reached almost 100% within 6 days. Further, the mortality rate of bradyzoites at 37°C showed the highest transition under aerobic and the lowest transition under anaerobic conditions. Mortality rates of bradyzoites stored at 4°C under different aerobic conditions were less than 10% on day 22 and gradually increased to 10%–16% on day 30. Mortality rates of bradyzoites stored at 4°C were not markedly different among different aerobic conditions. Effects of different concentrations of DMSO on bradyzoite viability are shown in Fig. 2. No difference in mortality was seen between 0 and 2% DMSO. However, DMSO was highly toxic to bradyzoites at concentrations of more than 5%. The effect of herbicides on bradyzoite viability and morphology was thus evaluated using 2% DMSO in PBS, incubation at 4°C and aerobic conditions.

Effect of herbicides on mortality and morphology of bradyzoites

Effects of different herbicide (30 mg)

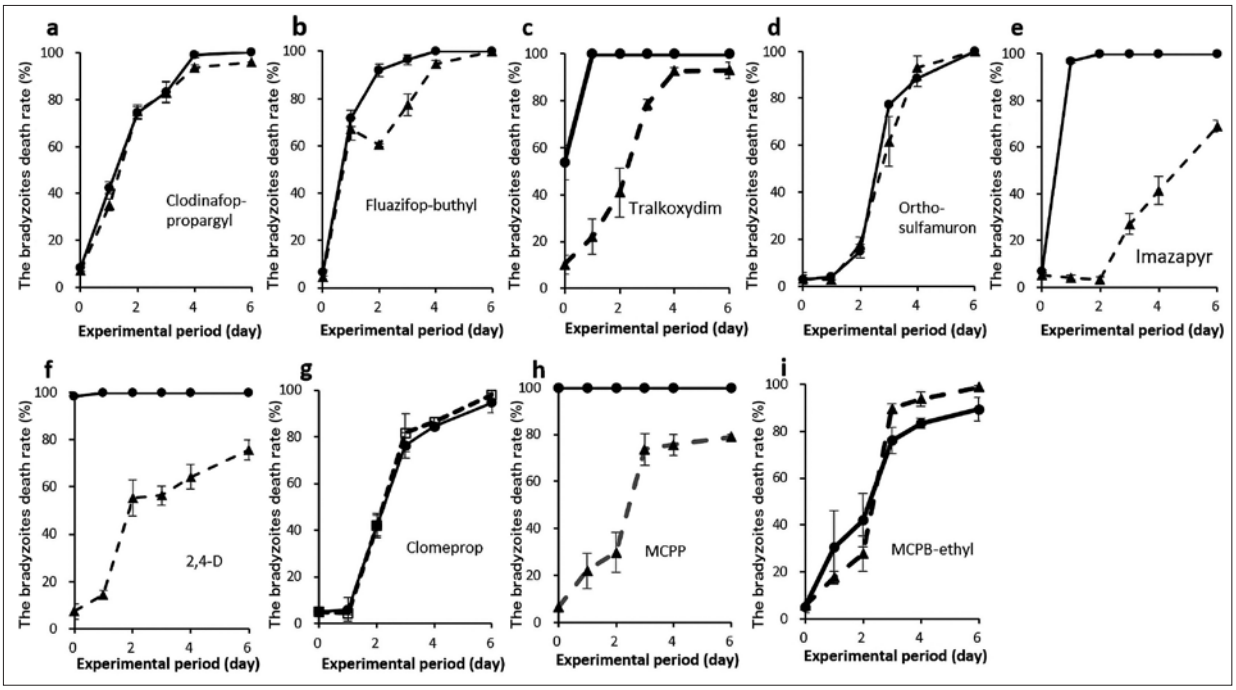


Fig. 4. The bradyzoite mortality effects of supernatants derived from suspensions of acetyl-CoA carboxylase (ACC) inhibitors (a, b, and c), acetolactate synthase (ALS) inhibitors (d and e), and auxin-like herbicides (f, g, h, and i). Solid lines indicate supernatants derived from 30 mg herbicide suspensions, while broken lines indicate supernatants derive from 3 mg herbicide suspensions.

suspensions on bradyzoite mortality are shown in Fig. 3. Effects of nine supernatants derived from herbicide (3 and 30 mg) suspensions that were effective against *Sarcocystis* bradyzoites are provided in Fig. 4. Mortality rates of bradyzoites in 2 % DMSO-PBS was less than 10 % at the end of the examination period. The pH of herbicide suspensions and blank were maintained at 7. Treatment with nine herbicides in three herbicide groups–ACC inhibitors (clodinafop-propargyl, fluazifop-butyl, and tralkoxydim), ALS inhibitors (orthosulfamuron and imazapyr), and auxin-like herbicides (2,4-D, clomeprop, MCPP, and MCPB-ethyl)–significantly increased mortality in bradyzoites within four days. (Fig. 3) Bradyzoite mortality at 3 and 30 mg supernatant of these 9 herbicides varied. (Fig. 4) Tralkoxydim, imazapyr, 2,4-D, and MCPP showed less mortality than bradyzoites than observed at 3 mg than 30 mg, but the other 5 herbicides showed no significant difference at the 2 concentrations.

The morphological changes in bradyzoites treated with 10 herbicides are shown in Fig. 5. Morphology of bradyzoites incubated with a blank solution is shown in Fig. 5A. Live bradyzoites in blank solution were transparent with a slight bright green color. The cytoplasm and nuclei of dead bradyzoites were stained dark blue and light blue, respectively. Dead bradyzoites did not exhibit marked morphological changes and maintained a crescent moon shape.

The effect of 30 mg suspensions of ACC inhibitors on bradyzoite mortality is shown in Fig. 3A, while that of the supernatant derived from 30 and 3 mg suspensions of ACC inhibitors on bradyzoite mortality is shown in Fig. 4a, b, and c. Mortality of bradyzoites incubated with 30 mg tralkoxydim suspension or its supernatant was approximately 50% immediately after treatment. Mortality did not significantly increase upon incubation with 30 mg suspensions of clodinafop-propargyl and fluazifop-butyl or their supernatants on day zero but increased to 90%–100% on day 4. Bradyzoites incubated with

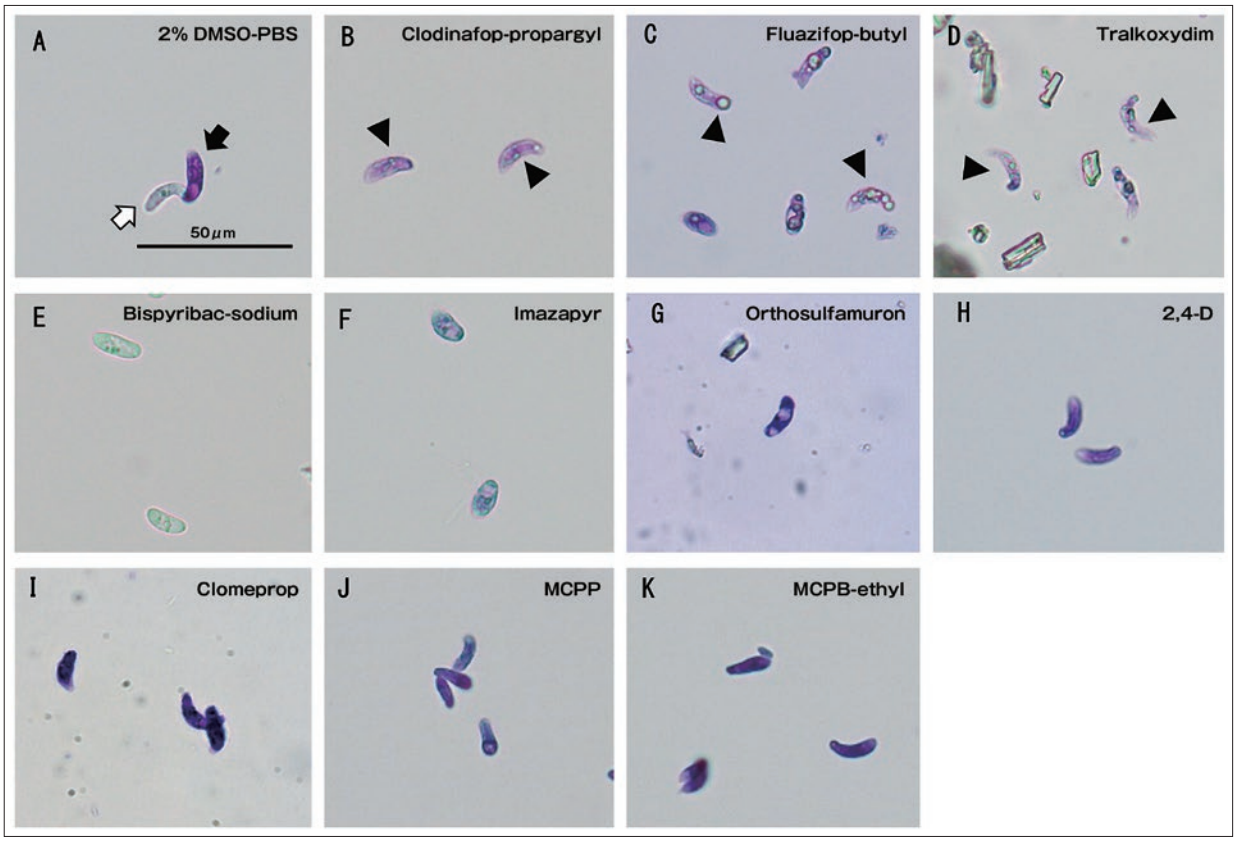


Fig. 5. Microscopy-based image of bradyzoites after treatment with different herbicides. White arrows indicate live bradyzoites, black arrows indicate dead bradyzoites, and the arrowhead indicates vacuole-like structures.

clodinafop-propargyl (Fig. 5B) and fluazifop-butyl (Fig. 5C) exhibited multiple vacuole-like structures. Bradyzoites incubated with tralkoxydim (Fig. 5D) exhibited coarsened and atrophied membranes and multiple vacuole-like structures.

Mortality of bradyzoites incubated with the 30 mg suspension of orthosulfamuron and imazapyr (ALS inhibitors) were 75% and 100%, respectively, on day one. In contrast, incubation with bispyribac-sodium did not affect mortality during the examination period (Fig. 3B). Mortality of bradyzoites incubated with supernatant derived from 30 and 3 mg suspensions of orthosulfamuron (Fig. 4d) was low on day one but gradually increased to approximately 90% on day 4. Mortality of bradyzoites incubated with supernatant derived from the 30 mg imazapyr suspension (Fig. 4e) was like bradyzoites incubated with 30 mg imazapyr suspension (Fig. 3B). The shape of bradyzoites was affected by incubation with suspensions of bispyribac-sodium (Fig. 5E) and imazapyr (Fig. 5F). The bradyzoites incubated with orthosulfamuron exhibited only a few vacuole-like structures (Fig. 5G).

Morphology and mortality of bradyzoites were not significantly affected upon incubation with inhibitors of photosynthesis, protox (Fig. 3C), carotenoid synthesis, and VLCFA synthesis (Fig. 3D).

The mortality rates of bradyzoites incubated with auxin-like herbicides (Fig. 3E) were high. Mortality of bradyzoites incubated with 30 mg suspensions of 2,4-D and MCPP suspensions (Fig. 3E) and their supernatants (Fig. 4f and h) were 100% on day 0. Mortality of bradyzoites incubated with clomeprop and MCPB-ethyl (Fig. 3E, 4g and i) were low immediately after incubation

but gradually increased to approximately 90% on day 4. The nucleus of bradyzoites incubated with all auxin-like herbicides was not visible, and bradyzoites were uniformly stained blue and did not exhibit a significant change in shape (Fig. 5H–K).

DISCUSSION

ACC inhibitors, ALS inhibitors, and auxin-like herbicides all display mortality effects on bradyzoites. This *in vitro* method enables screening of drugs against *Sarcocystis* bradyzoites. No established culturing methods have been developed for this species. Direct responses of bradyzoites to herbicides were thus observed. Bradyzoites may have differential organelles or metabolism targeted by herbicides. Further, *Sarcocystis* bradyzoites need herbicide targets for viability.

OECD guidelines require a concentration reaching the saturation concentration when assessing the effects of chemical substances[31]. Therefore, a solution containing 30 mg that exceeds saturation of all herbicide, and 1/10 of the saturated concentration of 3 mg was used as test suspensions/solutions. Dose-response of herbicides for bradyzoite mortality was different among herbicides. The sensitivity of bradyzoites to herbicides demonstrated different dose responses. Herbicide solutions need to be quantified and mortality of bradyzoites verified.

A previous study on ACC inhibitors reported that clodinafop, quizalofop, haloxyfop, and fluazifop were effective at inhibiting growth and replication of *T. gondii* tachyzoite, and that clodinafop was the most potent inhibitor[42]. However, clethodim, cethoxydim, and sethoxydim were not effective against *T. gondii*. Clodinafop-propargyl, fluazifop-butyl, and tralkoxydim affected mortality and morphology of *Sarcocystis* bradyzoites. ACC inhibitors exert herbicidal effects by inhibiting fatty acid synthesis catalyzed by ACC in the plastid. Fatty acid synthesis is catalyzed by fatty acid synthase type I (FAS I) and FAS II[35]. FAS I catalyzes the synthesis of fatty acids in eukaryotic cytoplasm; FAS II catalyzes synthesis of fatty acids in plastids of plants and algae. The type II fatty acid biosynthetic pathway is reported in *Plasmodium* and *Toxoplasma*[36,39]. *Sarcocystis* belongs to the same phylum as *Plasmodium* and *Toxoplasma*, and ACC inhibitors might target the apicoplast in *Sarcocystis*. However, the mechanism of action of ACC inhibitors is still unclear. ACC inhibitors do not target ACC metabolic pathways in malarial parasites because infectivity of asexual ACC gene knockout of malarial parasites is similar to wildtype malarial parasites[17]. Thus, despite a phylogenic connection to *Plasmodium* and *Toxoplasma*, ACC inhibitors may not target the ACC metabolic pathway in *Sarcocystis*. However, vacuolar structures not stained with methylene blue were observed in bradyzoite cytoplasm after treatment with ACC inhibitors. ACC inhibitors may target intracellular organelles of bradyzoites to increase secretion and form vacuolar structures.

Various ALS inhibitors differentially affect survival and morphology of bradyzoites. Pyrimidinyl benzoate and sulfonylurea herbicides inhibit ALS, involved in the synthesis of branched-chain amino acids in mitochondria and chloroplasts. Imidazolinone inhibits ALS by binding to pyruvate. These compounds exert herbicidal effects by disrupting protein synthesis and inhibiting cell growth and DNA synthesis[15]. Limited studies on proliferation inhibition of ALS inhibitors against Apicomplexa, and on infectivity and drug susceptibility of Apicomplexa are available.

Bradyzoites of *Sarcocystis* species have 22 subpellicular microtubules originating at evenly spaced intervals around the polar ring. These microtubules extend posteriorly immediately beneath the inner membrane complex for about 1/2 to 2/3 the length of the zoite[9]. The function of subpellicular microtubules is unclear, but is thought to provide structural integrity for the

shape of the bradyzoite. Alveolar endogenous membrane protein GAPM1 for maintaining microtubule stability of *Toxoplasma* tachyzoites. Degradation of GAPM1a caused subpellicular microtubule disorganization and subsequent depolymerization. These changes in the cytoskeleton led to parasites becoming shorter and rounder[18].

In this study, imazapyr and bispyribac-sodium significantly affected the morphology of bradyzoites. ALS inhibitors might affect metabolism of proteins, such as GAPM1 and subpellicular microtubules that constitute the cytoskeleton. Further, imazapyr and orthosulfamuron increased mortality of bradyzoites but bispyribac-sodium did not show this effect. Each ALS inhibitors differentially affected survival and morphology of bradyzoites. These results suggested that each ALS inhibitor has a unique mode of action against *Sarcocystis* bradyzoites.

Treatment with inhibitors of protox, carotenoid synthesis, and VLCFA synthesis did not affect the mortality of bradyzoites. Ponazuril and diclazuril are triazine-based antiprotozoal agents, like atradine, used in the treatment of EPM caused by *S. neurona*[6,11,12,25,28]. These drugs act on the apicoplasts of *Sarcocystis* but modes of action remain unknown, ponazuril inhibits merozoite production in cell culture by more than 90% and 95% at concentrations of 1 and 5 μg/mL, respectively[24]. Triazine-based drugs target apicoplasts, and we hypothesized that atrazine might be effective against bradyzoites in different stages from oocyst and merozoite. However, atrazine did not affect viability or morphology of bradyzoites. Thus, triazine-based drugs target metabolic processes in a stage-specific manner, and that the drug is metabolized only in a living body or specific cell.

The auxin-like herbicides, MCPP and 2,4-D exert a substantial effect on bradyzoites mortality. Auxins acidifies the cell wall by activating H+-ATPase and potassium ion channels, and inducing cell elongation by stress relaxation[34,38]. Additionally, 2,4-D alters the integrity of cell membranes and inhibits enzyme complexes involved in electron transfer, stress responses to reactive oxygen compounds, and oxidative phosphorylation in non-target species[7]. High mortality of bradyzoites after treatment with MCPP and 2,4-D may be attributed to auxin-like activity on ion channels expression on bradyzoite cell membranes. Treatment with clomeprop and MCPB-ethyl gradually increased mortality of bradyzoites, with 100% mortality observed at the end of the experimental period. Clomeprop is converted into 2-(2,4-dichloro-3-methylphenoxy) propionic acid (DMPA)—a metabolite produced during allyl acylamidase-catalyzed hydrolysis of acylamide in plants—which enhances auxin activity[37,40]. MCPB-ethyl is metabolized in plants, to MCP that promotes β-oxidation of the side chain and exerts herbicidal effects through the same mechanism as 2,4-D. Thus, MCPB-ethyl exhibits a weak herbicidal effect in plants that do no exhibit β-oxidation[27]. Bradyzoites may also be capable of acylamide hydrolysis and β-oxidation.

ACC inhibitors and auxin-like herbicides are reported to be effective against *Plasmodium* and *Toxoplasma*[4,8,19,20,23,42]. This study demonstrates that ALS inhibitors are also effective against *Sarcocystis* bradyzoites. Chemical properties of herbicides and their mode of action can be inferred based on affected functions in bradyzoites. Some ACC inhibitors and ALS herbicides have similar chemical structures or modes of action, but exhibit differential antiparasitic activities.

Sarcocystis shows specific metabolic functions in each life stage. Further studies in additional life stages are needed to elucidate the biological properties of *Sarcocystis* and develop therapeutic agents.

ACKNOWLEDGMENTS

We thank Dr. Tsutomu Omatsu (Tokyo University of Agriculture and Technology, Tokyo, Japan) and Dr. Shuetsu Fukushi (National

Institute of Infectious Diseases, Tokyo, Japan) for useful suggestions. We would like to thank Enago (www.enago.jp) for English language editing.

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