Sudan Red 7B, a Dye Marker for *Coptotermes formosanus*[1,2]

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ABSTRACT

Of 9 dyes fed to workers of *Coptotermes formosanus*, only oil soluble Deep Black BB and Sudan Red 7B stained the termites sufficiently and were retained long enough to be useful as markers. Workers fed Red 7B, however, absorbed the dye faster, retained the dye longer and had a lower mortality rate than those fed Deep Black BB. Red 7B was found in the fat bodies, mid- and hindguts, muscles, brain, and in the protozoa, but not in Malpighian tubules and tracheal matrix.

The density of the termites in the staining chamber seemed to affect the depth of staining and the numbers of protozoa in the termite. The termites stained at a density of 900 individuals per petri dish stained more deeply and better than those stained at 100 individuals per petri dish. Moreover, the numbers of protozoa in the termites stained at the 900 termite density were higher than in termites stained at 100 per petri dish.

Red 7B demonstrated all the characteristics required for a suitable marker for *C. formosanus*.

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is known to exist in large colonies (Tamashiro et al. 1980). Populations of such insects can be enumerated and followed by using the "Lincoln Index" (Ayre 1962; Southwood 1971), or by "marking, release and recapture methods" (Andrewartha and Birch 1967).

A basic prerequisite to the use of the marking, release and recapture method is the availability of a suitable marker and marking technique (Southwood 1971). According to Southwood, a suitable marker must: 1) not affect the longevity or behavior of the animals; 2) be recognizable during the experiment; and 3) not change the behavior of the colony towards the marked animal.

Although radioactive tracers have been used to mark termites (Gosswald and Kloft 1963), they have generally proved to be unacceptable because the tracer was passed from one termite to another through trophollaxis or grooming. An internal dye which makes a termite easily identifiable and which could not be passed from termite to termite was required. In addition, this dye should remain identifiable for at least 20-30 days so that the mark, release and recapture method could be used.

This study was made to select a suitable dye and to determine the effects of the selected dye on *C. formosanus* workers and their symbiotic protozoa.

MATERIALS AND METHODS

1). *Screening of Dyes*

The oil soluble dyes Yellow 3G, Orange R, Sudan Red 7B, and Deep Black BB, produced by BASF, Wyandotte Corp., and the water soluble dyes; Genacryl Red 4B

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produced by General Aniline and Film Corp.; Anthraquinone Green GNN by E.I. DuPont de Nemours and Co., Inc.; Eastacryl Blue 2R by Eastman Chemical Products Inc.; Brilliant Green by Hartman-Leddon Co.; and Calco Blue of an unknown source were tested.

A 1% solution of each of the dyes was made up in acetone. Two milliliters of the dye-acetone solution were drawn with a serological pipette and introduced into a 100 × 20 mm petri dish lined with 2 sheets of 9-cm filter papers (Whatman's No. 1). Two pieces of applicator sticks 7.5 cm in length, were placed beneath the filter papers to provide space for the termites.

The petri dish with the dyed filter paper was left open for at least 20 h to permit complete evaporation of the acetone. This was done in the laboratory at a mean temperature of 24.4°C with a range of 22.5° to 26.3°C and an average relative humidity of 56% with a range of 38% to 62%. At the end of the 20 h period, the filter papers were well stained and dry.

Two ml of sterile distilled water were added to the dyed paper to provide moisture for the termites just before introducing 50 workers into each dish. The dyed filter papers served as food for the termites during the period of marking. There were 3 replicates for each dye. The control was treated the same way except without the dye.

The termites were held in the petri dish and allowed to feed on the stained papers for 10 days. On the 10th day, the termites were transferred to a new petri dish containing unstained filter papers. The termites were observed daily to ascertain acquisition and retention of the dye and mortality. Two ml of sterile distilled water were added to each petri dish every 4 days to prevent desiccation. Dead termites were removed from the petri dish each day.

2). *Effects of Red 7B*

Since Red 7B appeared to have the characteristics required of a good termite marker, several additional tests were conducted to observe its effects in greater detail. The dye was dissolved in acetone at concentrations of 0.5, 1, 1.5, 2, and 2.5%. Two ml of the desired concentration of the dye were distributed evenly on the filter papers in petri dishes as described earlier. Three replications were prepared for each concentration and 50 workers were placed in each dish. The workers were exposed to the dye for 13 days and then transferred to a new petri dish containing unstained filter paper. The controls were set up in the same way except that the dye was not introduced. Daily observations were made for mortality and dead termites were removed at the time of observation. The data were transformed using $\sqrt{x + 0.5}$ and subjected to analysis of variance.

In addition to observations on mortality, micro-sections of stained termites were made to ascertain the distribution of the dye in the tissues. However, conventional techniques, i.e., embedding in paraffin, could not be used, since the procedures of fixing, dehydrating, and embedding in paraffin would have eliminated the dye from the tissues. A freezing technique was used to section the termites.

A dyed worker was fixed for 1 h in 10% formaldehyde solution. The fixed termite was quick frozen in a cryostat. This generally took 1 or 2 minutes. When necessary, freon-11 was used to enhance freezing or to lower the temperature for better sectioning. The specimen was then mounted on a stage, sectioned, and the sections were taken up with a clean microscopic slide and examined.

Since feeding the dye to the termite also exposed the symbiotic protozoa in the hindgut of the termite to the dye, a study was made to assess the effect of Red 7B on the

protozoa. In addition, since preliminary observations seemed to indicate that the density at which termites were held in the staining chamber affected the termites uptake of dye, population density was included as a variable.

A high or low population density consisting of 900 or 100 termites, respectively, was placed in similar sized petri dishes (78.5 cm^2) containing Red 7B stained filter papers. There were 3 replicates for each density. The termites were held for 10 days and each day, 3 workers were selected at random and dissected to sample the protozoa. A microsyringe method employed by Lai (1977) was used to count the protozoa. Three aliquots of 2 μl each were drawn from the suspensions of protozoa and counted. The data were subjected to analysis of variance for factorial design and the means were compared using Duncan's multiple range test (SAS 1982)

RESULTS AND DISCUSSION

1). *Screening of Dyes*

Among the 9 dyes tested, Orange R caused the highest mortality (62.4%) and Red 7B caused the lowest mortality (Table 1). The affected workers were sluggish and their abdomens were shrunken. Their abdomens shrank dorso-ventrally and antero-caudally so that it appeared round when viewed from the dorsal aspect. This type of abdominal shrinkage is characteristic of starving workers. The termites became sluggish 24 h after exposure to Orange R and 48 h after exposure to Anthraquinone Green GNN, Genacryl Red 4B and Brilliant Green. With Eastacryl Blue 2R, however, the symptoms did not appear until 7 days after the exposure. The staining ability of the dyes was not correlated with their toxicities.

C. formosanus workers were not stained by Yellow 3G, Orange R, Brilliant Green and Calco Blue although the termites fed on the dyed papers. Workers were lightly stained by Genacryl Red 4B, Anthraquinone Green GNN and Eastacryl Blue 2R. These dyes, however, did not penetrate the gut and stain other internal tissues. The remaining two dyes, oil soluble Deep Black BB and Sudan Red 7B, both deeply stained the termites.

Workers exposed to the Deep Black BB were not noticeably stained until 6 days after exposure and were deeply stained after 9 days. Workers exposed to Red 7B, however, were obviously stained by the third and and were deeply stained by the fifth day after exposure. Actually, the alimentary tract of the termite was outlined in red within hours after the termites were fed the dyed paper. Both dyes were visible in the workers for as long as 30 days after they stopped feeding on the dyed filter paper. Termite mortality, however, was lower in Red 7B although the difference was not statistically significant. Termite mortality in the Red 7B treatment was no different than in the controls.

The additional test conducted with concentrations of Red 7B as high as 2.5% showed that the mortalities in the treated termites were not significantly different ($P>.05$) from the controls. Moreover, there were no visible differences in staining among the different concentrations at the end of 13 days. The results indicated that Red 7B was innocuous to *C. formosanus* and could be used in the field.

Within the termites, it could not be determined whether the dye was absorbed in the midgut which would indicate that the dye was soluble in the midgut juices, or whether the dye was absorbed in the hindgut after the protozoa predigested the paper containing the dye. The dye was found in the fat bodies, mid- and hindguts, muscles, brain, and in the protozoa. Although the workers appeared completely stained, the dye was not present in the Malpighian tubules and tracheal matrix. That the

TABLE 1. Average mortality caused by 9 dyes fed to *Coptotermes formosanus* workers for 10 days.

Days	Test Dyes									
	Orange R	Brilliant Green	Estacryl Blue 2R	Genacryl Red 4B	Anthraquinone Green GG	Deep Black BB	Yellow 3G	Calco Blue	Control	Red 7B
1	5.3	3.3	2.3	3.7	6.0	3.3	4.3	2.0	2.3	0.7
2	3.3	1.3	0.7	1.7	1.0	1.7	0.7	1.0	1.7	0.7
3	4.0	0.3	0.0	1.0	2.3	1.0	2.0	0.7	2.3	2.3
4	3.3	3.3	1.3	0.3	3.7	0.3	1.3	0.7	0.7	0.0
5	1.0	2.0	0.7	0.3	1.3	0.3	0.3	0.7	0.7	0.3
6	3.3	4.7	1.0	10.0	1.7	0.0	0.3	0.7	0.3	1.7
7	1.7	4.3	6.0	3.7	1.3	3.7	1.3	1.7	0.3	1.3
8	1.3	4.0	5.3	3.0	0.7	3.7	0.7	1.0	0.3	1.3
9	3.3	4.3	2.7	1.0	1.7	2.3	1.7	1.3	0.3	0.3
10	4.7	1.3	7.3	0.7	1.7	1.3	3.0	0.7	0.7	0.7
Total	31.2	28.8	27.3	25.4	21.4	17.6	15.6	10.5	9.6	9.3
% Mortality	62.4	57.6	54.6	50.8	42.8	35.2	31.2	21.0	19.2	18.6
Mean*	3.12	2.88	2.73	2.54	2.14	1.76	1.56	1.05	0.96	0.93

*Means with a continuous underscore are not significantly different from each other at the 5% level.

TABLE 2. Average number of protozoa per 2 μl sample for workers fed the dye, Red 7B, for 10 days. Two densities of termites, 900 and 100 per Petri dish, were treated.

	900 Termites		100 Workers	
Days	Stained	Unstained	Stained	Unstained
1	33.7	25.9	31.0	34.3
2	45.7	28.1	38.3	24.7
3	27.3	30.6	26.6	31.0
4	28.4	34.0	26.0	30.3
5	30.0	36.3	11.4	41.0
6	22.3	31.7	16.0	41.0
7	28.4	37.7	17.1	24.3
8	14.7	30.0	12.7	22.0
9	25.0	31.0	11.7	40.3
10	17.3	23.7	8.7	8.3
Total	272.8	309.0	199.5	297.2
Mean*	27.3[a]	30.9[a]	20.0[b]	29.7[a]

*Means with the same superscript are not significantly defferent from each other at the 5% level.

Malpighian tubules were not stained was surprising since they must have been involved in the excretion of the dye. The excreta of the stained termites were red.

Spots of red were also seen in some protozoa but the protozoa generally did not take the stain uniformly. According to Kudo (1966), all three flagellates inhabiting *C. formosanus* lack cytostomes and rely on pseudopodia to ingest food. Food ingestion by these protozoa, therefore, was a form of phagocytosis. Observations revealed that there were differences in the amount of dye, i.e., food, phagocytosed by the three species of protozoa. There were also differences in the appearance of the dye in the protozoa.

In *Pseudotrichonympha grassii* Koidzumi, the dye appeared in spots in various parts of the body. The protozoa was not uniformly stained. In *Holomastigotoides hartmanni* Koidzumi, on the other hand, the dye was found to be more uniformly spread throughout the body. The dye particles in *Spirotrichonympha leidyi* Koidzumi, were very difficult to detect because of the minute size of this species. Sometimes, however, a tinge of red could be detected in some individuals.

Although the appearance of the dye was different in each of the 3 species of protozoa, it did not necessarily result from qualitative differences in the symbiotic functions of the protozoans. Termites defaunated and refaunated with only one of the several species protozoa occurring in the hindgut have survived (Mauldin et al. 1972) indicating that one protozoan species was sufficient to provide the required nutrients.

The dye did seem to reduce the total number of protozoa after about 10 days but the reduction was only statistically significant in those termites stained at a density of 100 termites per petri dish (Table 2). At a density of 900 termites per petri dish, the protozoan population of the termites fed the dye was similar to that of the control termites. That there was a significant reduction in the numbers of protozoa in the group stained at 100 per petri dish was surprising, since, the termites apparently were unaffected by the dye. There was no significant termite mortality, the workers

appeared normal and were apparently able to function normally. The reduction in the number of protozoa to the levels recorded apparently was not serious enough to significantly affect the termites.

It was also significant that termite density in the staining chambers seemed to affect the amount of dye picked up by the termites. The termites stained at high density, 900 termites per petri dish, were more deeply stained than those held at low density.

Why termite density affected the number of protozoa in stained termites and why the termites from the group held at high density stained deeper are not known. If density alone was a factor then the unstained termites held at low density should have also lost protozoa. This did not occur. Moreover, if the dye alone was affecting the protozoa, then the termites held at high density which had taken in more dye should have had less protozoa. It is probable that both factors, density and the stain had a cumulative effect which became more pronounced with time. Of these density was probably more significant.

C. formosanus may be a species that requires a large number of individuals per unit area to function effectively. With high termite densities, the individual insects would have more opportunity to feed each other, both orally and proctodaeally and there would be more termite to termite contact. This is a factor to be considered in laboratory experiments with this species.

This study therefore, has shown that the dye, Sudan Red 7B, has the characteristics required of a marker for *C. formosanus*. The selection of this dye has not only enabled an in-depth study of the cryptic behavior of this termite, but also facilitated experiments on the enumeration of termite populations.

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