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Evaluation of Non-Toxic Visible Dyes as Markers for *Microtermes obesi* and *Odontotermes lokanandi* (Blattodea: Termitidae)

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ABSTRACT

Workers and soldiers of *Microtermes obesi* Holmgren and *Odontotermes lokanandi* Chatarjee and Thakur (Blattodea: Termitidae) were force-fed on different concentrations of Nile blue-A and Sudan red-7B. Results showed that 0.5% Nile blue-A caused 100 % mortality in *M. obesi* on 15th day in *M. obesi* and on 9th day, whereas 0.5% Sudan red caused 100% mortality in *M. obesi* on 9th day in *O. lokanandi*, on 5th day *M. obesi* and *O. lokanandi* were released on baits containing different concentrations of Nile blue-A. *M. obesi* got colour at 100% relative humidity after day 10 at all concentrations, while at 92 and 76% relative humidity they got colour at high concentration only. However, *O. lokanandi* was found sensitive to Nile blue. *M. obesi* and *O. lokanandi* released on baits of Sudan red-7B, did not get any colour under all tested relative humidities. Nile blue-A and Sudan red at medium and low concentrations remained in bodies of more than 90% *M. obesi* for 25 and 10 days, respectively. After 60 days, maximum of 59.33% termites were blue with low concentration of Nile blue. All *M. obesi* stained with Sudan red were dead by day 60.

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Authors' Contributions
AS designed and executed the experiment, and wrote the article. MN and EH supervised the study. AM and AH statistically analyzed the data.

Key words
***Microtermes obesi, Odontotermes lokanandi*, nile blue-A, sudan red-7B.**

INTRODUCTION

Fruit trees, crops and buildings are seriously damaged by some subterranean termites. Their cryptic habits make study of their populations extremely difficult. Their nests or reproductive centers may be underground or in mounds on the surface, in the stumps or logs, or within or attached to the trunks or branches of trees. A colony may consist of a single centre or of several interconnected units (Nutting and Johnes, 1990). Study of the population demographics and foraging behavior of subterranean termites poses difficulties, due to the subterranean gallery system and the absence of a well-defined nest architecture that is separable from the surrounding soil matrix. The knowledge of the basic ecology and biology of subterranean termites is essential for the development of effective control strategies. A marking material is required for studies of population dynamics of subterranean termites under field conditions.

Marking termites effectively is difficult because of both their physical fragility and the very large numbers typically found in individual colonies, such that there is no consensus on the optimum marking material (Evans, 2000). An ideal marking material as durable, un-expensive, non-toxic, easily applied, and clearly identifiable; furthermore, the marker should not hinder the insect nor affect its normal behaviour, growth, reproduction, or lifespan (Hagler and Jackson, 2001). The use of fat-soluble histological dyes to mark termites has been frequently explored. The limited cuticle sclerotization of termites permits the use of histological markers such as Sudan red-7B, Nile blue-A or Neutral red (Su *et al.*, 1991; Evan, 1997). The use of Neutral red and Nile blue-A is most commonly reported (Grace and Abdallay, 1989; Evans *et al.*, 1998; Tsunoda *et al.*, 1999; Stanley *et al.*, 2001). In addition, other dyes have also been evaluated, for instance Sudan black, Sudan yellow, Sudan green, and Sudan red (Su and Scheffrahn, 1988; Grace, 1990; Salih and Logan, 1990; Evans, 1997).

Many researchers reported that the use of stains has several disadvantages: the insects have to ingest the stain diluted in aqueous solution or impregnated in filter paper, it is time-consuming, some of these substances accelerate termite mortality (Grace and Abdallay, 1990; Evans, 1997; Nobre *et al.*, 2007) and finally, these markers do not offer good visual contrast. The main disadvantages of dyes have been reported to be non-homogeneous colouration, variable fade-out, and unintended transfer to other individuals by cannibalism and trophallaxis (Haagsma and Rust, 1993; Thorne *et al.*, 1996; Curtis and Waller, 1997; Evans *et al.*, 1998; Suarez and Thorne, 2000). However, Nile blue-A has a record of success in several studies (Haagsma and Rust, 1993; Evans *et al.*, 1998, 1999; Marini and Ferrari, 1998;

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Tsunoda *et al.*, 1999; Evans, 2001; Stanley *et al.*, 2001).

Some studies have documented that the retention of dyes in or on termites varies significantly between both the type of dye applied and the termite species under investigation *i.e.*, Neutral red and Nile blue-A have proven valuable for long-term studies *i.e.*, 11 weeks for certain termite species, but not for others (Haagsma and Rust, 1993; Oi 2000; Su *et al.*, 1991). Lai (1977) was the first researcher, who used dye markers to estimate foraging population of three *Coptotermes formosanus* Shiraki colonies. Lai *et al.* (1983) screened nine histological dyes and identified Sudan red-7B as the most persistent and least toxic dietary dye marker for *C. formosanus*. Sudan red-7B caused delayed mortality, and with time, the dye faded sufficiently that it could not be seen in an increasing number of termites (Su *et al.*, 1983; Delaplane *et al.*, 1988). Sudan red-7B could safely be used with shorter 3 week release-recapture cycles with *Reticulitermes flavipes* Kall (Grace, 1989, 1990; Grace and Abdally, 1989). Neutral red was also identified by Salih and Logan (1990) as the most promising of 30 dyes listed as markers for *Microtermes lepidus* Sjostedt. Su *et al.* (1991) identified Nile blue as a safe and persistent marker for *R. flavipes.*

In the present study, we determined the efficacy of two dye markers, Nile blue-A and Sudan red-7B and proper concentrations of visible dyes in the bodies of *Microtermes obesi* Holmgren and *Odontotermes lokanandi* Chatarjee and Thakur (Blattodea: Termitidae).

MATERIALS AND METHODS

Biological stains

Nile blue-A (96%) and Sudan red-7B (95%) were evaluated as dye markers. These compounds were selected from biological stains that are used for dying animal tissues, lipids or cell granules. Acetone was used as solvent for the dyes.

Experimental termites

M. obesi and *O. lokanandi* were captured using NIFA-TERMAPS (Salihah *et al.*, 1993) from building of Directorate of Health Services, Capital Development Authority, Islamabad**.** The termites were brought to Entomological Laboratory, National Agriculture Research Center, Islamabad. The termites along with the soil and debris were passed through 5.0, 4.0 and 1.0 mm mesh sieves in regular sequence. After that the termites along with debris and soil were put on the inverted glass Petri dish placed on the apparatus set up by NIFA termite group consisting of a plastic tub (29.5 cm dia.) with inverted glass Petri dish (15.3 cm dia.). The termites without any extra particle fell down in the tub. The termites and debris on the Petri dish were frequently disturbed with a camel brush to collect all the termites in the tub. The Petri dish along with the remaining debris was gently removed and the termites were introduced in other glass Petri dishes (15.3 cm dia.), each having two same size filter papers moistened with distilled water and kept as stock termites in controlled temperature room *i.e.*, 27±3°C and 60±5% RH. Identification of termites was done using the key by Chaudhry *et al.* (1972).

Preparation of dye bait

Saw dust (100 g) were soaked with 100 mL of each of the dye solutions (0.125%, 0.25% and 0.5%). For control series 100 g of saw dust was soaked with acetone only following the techniques used by Su *et al.* (1983). For solvent evaporation, the dyed saw dust and the control series were kept at room temperature for 48 h. Each lot of stained and unstained saw dust was mixed with 1% of hot agar in the ratio of 2g: 3mL (w/v). Three Petri dishes were used for each treatment. Each Petri dish (5.3 cm dia) was half filled with stained bait of different concentrations and the other lot with unstained bait. 200 termites (180 workers and 20 soldiers) were introduced into each Petri dish containing dyed bait and were force-fed followed the technique described by Su *et al.* (1988). For control series 200 termites (180 workers and 20 soldiers) were released on unstained bait. All the experimental units were kept in desiccators at 92% relative humidity. The experiment was designed as completely randomized with three replications. Data was taken on daily basis. Percent mortality was corrected using Abbot's formula (1925). Statistical computation was performed using Co-State. Means were separated using Duncan's Multiple Range Test.

Visibility of dye markers in termite bodies at different relative humidities

The experiment was conducted to screen out the best relative humidity for staining termites. The relative humidities used were 100% (H_2O), 92% (Na_2Co_3) and 76% (NaCl). Saturated solutions of those salts were prepared in desiccators and kept covered at controlled temperature (28±2°C) and relative humidity (60±5%) for 48 h to maintain the required humidities. The glass Petri with Nile blue-A Sudan red TB and dye having baits at 0.5%, 0.25% and 0.125% concentrations (prepared as prescribed above), each with 100 termites (90 workers and 10 soldiers) were kept in desiccators. Colour of the termite bodies was observed daily; any clear change in the dye gained by termites was recorded. The number of survivors was recorded on a daily basis up to 15 days.

Retention test

A culture of termite (*M. obesi*) was collected from the field and acclimatized in the laboratory for 48 h. Three concentrations (0.5%, 0.25% and 0.125%) of Nile blue-A and Sudan red-7B dyes were prepared. In each concentration, a filter paper was soaked and then kept at room temperature for 48 h for solvent evaporation. A cluster of *M. obesi* was released onto each concentration and force-fed for 24 h. Fifty (50) stained worker termites from each concentration were selected at random and transferred to Petri dish (5.3 cm dia) containing un-dyed bait (prepared as above). In addition, five un-dyed worker and soldier termites were added to each Petri dish. There were three replicates for each treatment. Number of stained termites and survivors were recorded after 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 days. Statistical computing was performed using Co-Stat.

RESULTS

Toxicity of Nile blue-A

Figure 1A shows that mean mortality (%) in *M. obesi* after forced-feeding them on baits containing Nile blue-A was not different for high (0.5%) , medium (0.25%) and low (0.125%) dye concentrations ($P>0.05$) up to 5th day; mean mortalities in *M. obesi* on days 6-9 were found not different at medium and low concentrations ($P>0.05$), but significantly differed ($P<0.05$) from mean mortality recorded at high concentration (Fig. 1A). Mean mortalities recorded on days 10th to 15th were found significantly different ($P<0.05$) between high, medium and low concentrations.

Figure 1B shows that mean mortality (%) in *O. lokanandi* force fed on bait containing Nile blue-A was found not different ($P>0.05$) at high (0.5%), medium (0.25%) and low (0.125%) concentrations on the 1st day; however, on 2nd day mean mortalities in *O. lokanandi* recorded at medium and low dye concentrations were not different ($P>0.05$), but significantly different ($P<0.05$) from mean mortality recorded at high dye concentration (Fig. 1B). On days 3-5 mean mortalities recorded at high, medium and low dye concentrations were found significantly different ($P<0.05$) from each other.

Toxicity of Sudan red-7B

Figure 1C shows that mean mortalities (%) in *M. obesi* force fed on bait containing Sudan red-7B were found statistically not different ($P>0.05$) for three levels of dye concentration up to 3rd day. On day 4, 5 and 7, mean mortalities at high dye concentration were significantly higher ($P<0.05$) than at medium and low dye concentrations; there was no significantly difference ($P>0.05$) in mortalities at medium and low dye concentrations (Fig. 1C). On days 6, 8 and 9, mean mortalities in *M. obesi* at three dye concentrations were found significantly different ($P<0.05$) from each other (Fig. 1C).

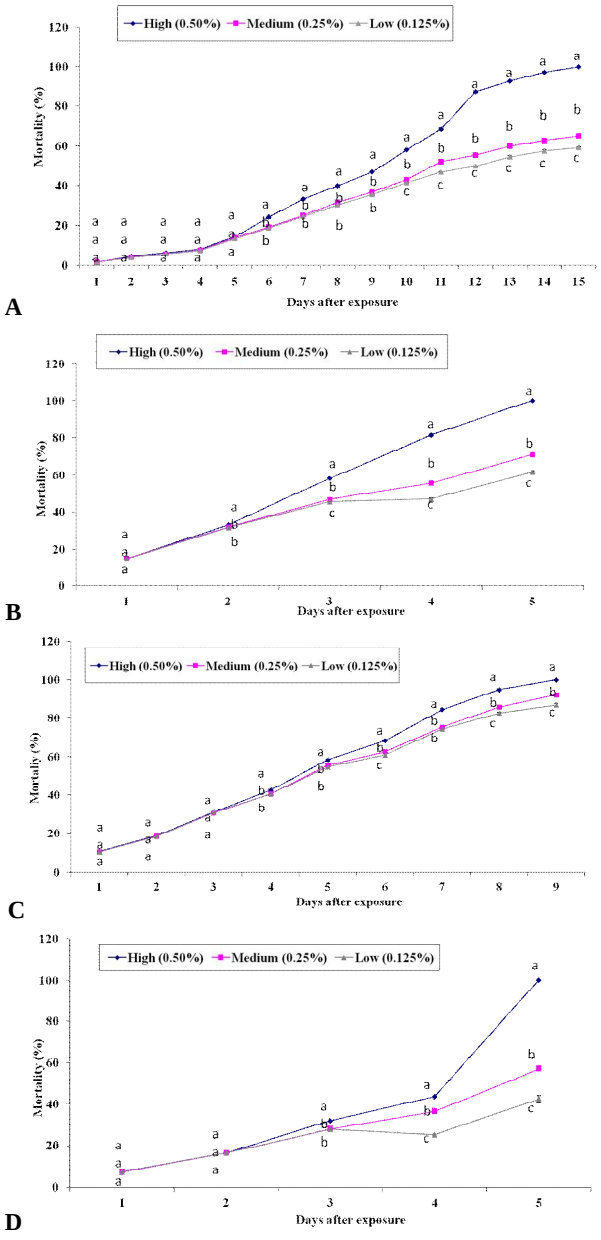

Fig. 1. Mortality (%) of *Microtermes obesi* and *Odontotermes lokanandi* after exposure to Nile blue-A and Sudan red-7B at 0.5% (high), 0.25% (medium) and 0.125% (low) concentrations. (A) *M. obesi* exposed to Nile blue-A. (B) *O. lokanandi* exposed to Nile blue-A. (C) *M. obesi* exposed to Sudan red-7B. (D) *O. lokanandi* exposed to Sudan red-7B.

Mean mortalities (%) in *O. lokanandi* force-fed on baits containing Sudan red-7B (at high, medium and low concentrations) were not different (P>0.05) amongst treatments up to 2nd day (Fig. ID). On day 3, mean mortality in *O. lokanandi* was significantly higher (P<0.05) at high dye concentration, while mortalities recorded at medium and low dye concentrations were found not significantly different (P>0.05). On days 4 and 5, mean mortality observed at high, medium and low dye concentrations was significantly different (P<0.05) amongst all treatments (Fig. 1D).

Visibility of dyes in termite bodies

When released on baits with 0.125, 0.25 and 0.5% concentrations of Nile blue-A *M. obesi* got colour under at 100% relative humidity after 10 days in all dye treatments. At 92% and 76% relative humidity they got colour at 0.5% dye concentration, but no colour was observed at 0.125 and 025% dye concentrations. *O. lokanandi* was more sensitive to dye than *M. obesi* in the same experiment. A greater percent of termites was found dead just after 4 days; the maximum longevity (4 days) was recorded only under at100% relative humidity.

When released on baits with 0.125, 0.25 and 0.5% of Sudan red-7B, *M. obesi* did not get any colour under any of the relative humidities (100, 92 and 76%) after 4 days. The Sudan red dye was found toxic to *O. lokanandi* at all relative humidities and there was no indication of red colouration in their bodies during their maximum longevity (24 h).

Retention of dye

When the dyed *M. obesi* were transferred to un-dyed attractive bait, more than 90% termites which had 0.25% and 0.125% concentration of Nile blue, retained dye marker in their bodies up to 25 days, and for 15 days after having 0.50% concentration of Nile blue. More than 90% termites which had 0.125% and 0.25% concentrations of Sudan red, retained dye marker in their bodies for 10 days, and for 5 days after having 0.50% concentration (Fig. 2). After 60 days, 59.33% and 42% termites retained dye marker after having 0.125% and 0.25% concentrations of Nile blue, respectively. All termites stained with Sudan red were dead by day 60.

DISCUSSION

In the present study Nile blue-A was found non-toxic for *M. obesi* and was retained well for maximum period of time, while *O. lokanandi* was found more sensitive than *M. obesi* to Nile blue-A. These results agree with those of Su *et al.* (1991), who identified Nile blue-A as a safe and persistent marker for *Reticulitermes flavipes* Kall. Nile blue-A at 0.25% concentration against *Heterotermes indicola* (Wasmann) was found as non toxic long persistent and best marker in laboratory, as well as in the field (Salihah *et al.,* 1994, 1995, 1996, 1997).

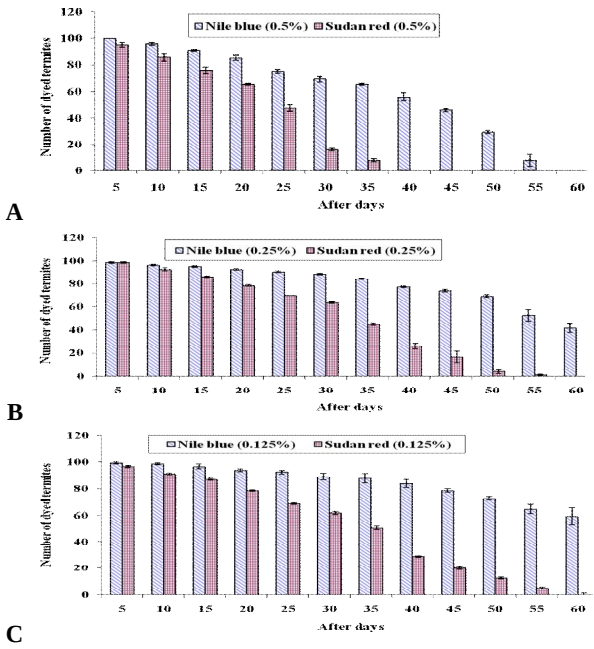

Fig. 2. Percent of dyed termites (*Microtermes obesi*) after specified number of days, at three concentrations.

The results showed that *M. obesi* was also more resistance to Sudan red-7B as compare to *O. lokanandi*, but Sudan red-7B caused 100% mean mortality in *M. obesi* after 9 days at high concentration, and 86.89% mortality at lower concentrations. Sudan red-7B caused 100% mean mortality in *O. lokanandi* at high concentration after 5 days, and 42.53% mortality at low concentrations. Many researchers observed that Sudan red had been considered as a suitable biological stain (Lai, 1977; Su *et al.,* 1988; Delaplane *et al.,* 1988; Delaplane and La Fage, 1989) for *Coptotermes formosanus* Shiraki, but not appropriate for making *R. flavipes*. Grace and Abdally (1989) demonstrated that Sudan red-7B could safely be used with shorter release and recapture cycle with *R. flavipes*. Grace and Abdallay (1989) demonstrated that low concentrations of Sudan red-7B are rapidly excreted by *R. flavipes*, and that extended feeding periods result in high mortality.

The results on the effect of Sudan red-7B at different relative humidities *i.e.*, 100, 92 and 76% showed that the dye was toxic to *O. lokanandi* and there was no

indication of red colouration in their bodies during their maximum longevity (24 h). Sudan red-7B was found to reside the longest in and cause the least mortality of the Formosan subterranean termite, *C. formosanus* (Lai *et al.*, 1983), it has been successfully used for estimating the population size of *C. formosanus* field colonies (Lai, 1977). The results support the data that Nile blue-A and Neutral red can persist for different times in different species and these different species in turn have different tolerances to these substances (Evan, 1997).

The decrease in number of dyed termites with the passage of time in the present study was due to the mortality of the termites, but not due the trophallactic transfer of dye. Salihah *et al.* (1994, 1995, 1996, 1997) revealed that Sudan red-7B at 0.25% concentration was non toxic to *H. indicola* and gave prominent pink colour to termite, but its retention period in field (42 days) was less than that of Nile blue-A (1 year and 3 months). Nile blue-A retained in *R. flavipes* and *C. formosanus* species throughout the 15 days period and did not cause significant mortality (Su *et al.*, 1991). Su *et al.* (1983) mentioned that the amount of Sudan red-7B in termites decreases sharply immediately after the termites stopped feeding on the stained paper.

In the present study Nile blue-A was found non-toxic for *M. obesi* and was retained well for maximum period of time, whereas Sudan red-7B caused comparatively more mortality. However, *O. lokanandi* was found sensitive to both Nile blue and Sudan red dyes. Nile blue-A (0.125%) caused lower mortality in *M. obesi* and was retained well for eight weeks in more than 59% termites; it can be recommended for use against *M. obesi* and may be used for long studies. Sudan red-7B caused comparatively more mortality, but can be used in short-term studies.

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