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An effective attractant for the pinewood nematode vector *Arhopalus rusticus* in South Korea

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ABSTRACT
The longhorn beetle, *Arhopalus rusticus*, is an agriculturally important quarantine pest due to its ability to carry the pine wood nematode (*Bursaphelenchus xylophilus*), which causes pine wilt disease (PWD). Reduction or elimination of the vector of *B. xylophilus* would be regarded as an effective measure towards the goal of controlling PWD. Since the trapping method is popularly used to monitor and kill the vectors of pine wood nematode recently, the effective and cheap attractant would be needed. The present study describes the creation of an attractant for *A. rusticus* and the development of a quick, easy way to evaluate the effectiveness of attractants.

Keywords: *Arhopalus rusticus*, pine wood nematode, beetle attractant, evaluation method.

1. Introduction

Arhopalus is a Northern Hemisphere cerambycid genus with about 25 species worldwide. Its main hosts are coniferous plants [1]. Linit listed *A. rusticus* carrying *Bursaphelenchus xylophilus* dauer larvae in Japan and North America [2]. Later, throughout Russia, *A. rusticus* was observed to act as a vector of *Bursaphelenchus spp.* [3]. In China, *A. rusticus* carries *Bursaphelenchus mucronatus,* and has been described as an important class of quarantine pest since it transmits the nematode to pine wood [4]. In Australia, *A. rusticus* is not a native species, but it has recently been found there on dead pine trees, where it may act as a vector to spread nematodes [5]. *Arhopalus* spp. cause damage to sickly and dead pines, affecting the timber trade between Australia and New Zealand, as well as other countries [1]. *A. rusticus* was also recently introduced into Argentina, in 2004[6]. In Korea, *A. rusticus* is included in the Korea forest research institute's morphological descriptions of pinewood nematode vectors [7].

Trapping methods have been used to monitor and kill vectors of pine wood nematode. These methods have been regarded as effective measures to control pine wilt disease (PWD), and this has encouraged further research and development. In recent years, such trapping methods have became more popular, and some beetle attractants have been developed into commercial products [8-11]. Blended with ethanol, both the monoterpene α-pinene and turpentine oil (α-pinene, β-pinene, camphene, limonene, β-phellandrene, myrcene), have been reported to be effective beetle attractants, based on comparisons of the attractants, mostly through evaluations in the field [11-13]. In our experience, the density of beetles has been distinctly different in different trapping sites, even when traps are spaced only 2 m apart. Differences in the number of beetles trapped can be substantial, depending on sunlight intensity, wind direction, and density of beetle hosts (weak pine). In order to better control for these various environmental factors, a simple and credible evaluation method is required. In this report, we have developed not only an effective *A. rusticus* beetle attractant, but also an easy, quick, convenient, and reliable method for the comparison and evaluation of attractants. This in vitro method is expected to save time and resources, facilitating the screening of multiple attractants, compared with traditional field tests.

2. Materials and Methods

2.1. Preparation of attractants

The experimental attractant was prepared by adding Tween 20 (Samchun Pure Chemical Co., Ltd, KOREA) at a proportion of 1:5 to the bait, which consisted of 20% (v/v) turpentine oil (Junsei Chemical Co., Ltd, Japan), 50% (v/v) ethanol (Samchun Pure Chemical Co., Ltd, KOREA) at a proportion of 1:5 to the bait, which consisted of 20% (v/v)turpentine oil (Junsei Chemical Co., Ltd, Japan), 50% (v/v) ethanol (Samchun Pure Chemical Co., Ltd, KOREA), 10% (w/v) benzoic acid (Sigma Chemical Co., USA), and 20% (v/v) aqueous extract of pine tree. The attractant was then poured into a 50 ml plastic tube with a cotton core and 5 holes (diameter of 0.5 cm) in the cap. The positive controls consisted of turpentine oil and α-pinene, each mixed with 95% ethanol at a ratio of 1:1. The control was 50% ethanol. Each control mixture was poured into the same type of tube as the attractant. The attractants in tubes were placed in a 26 °C incubator for 5 days, and weighing the weight of each tube every day, and the diffusion rate each day was calculated.

2.2. Collection of beetles
Beetle traps were purchased from Enjoy Technology Co., Ltd, Fujian, China. Each trap includes a plastic tube with the experimental attractant. This tube was placed in the bottom of the trap, which was then hung from trees in the pine forest of Chungnam National University in South Korea. From 8-Aug to 5-Sep, 2013, beetles were collected from the traps once per week. Beetles identified as *Arhopalus rusticus* were retained for further analysis; all other insects found in the traps were discarded.

2.3. Measurement of the effects of the attractants on beetles
The attractant was dropped into a plastic cap with cotton (shown in Fig. 1-A), and beetles were divided into two groups for testing. The first group was comprised of seven males and the second group was comprised of seven females. The device shown in Fig. 1-B was used to detect the effects of the proposed attractant; the unbent arm was connected to a blank cage treated with water only, and the bent arm was connected to a cage treated with the attractant. A gentle breeze from the fan served to transfer the odor of the attractant to the entrance. After allowing the beetles to get into the entrance for 5 min, the unbent arm was rotated 90 degrees relative to the bent arm, then the device was stood on the two cages. The number of beetles in the attractive cage was recorded, and the attraction rate was calculated.

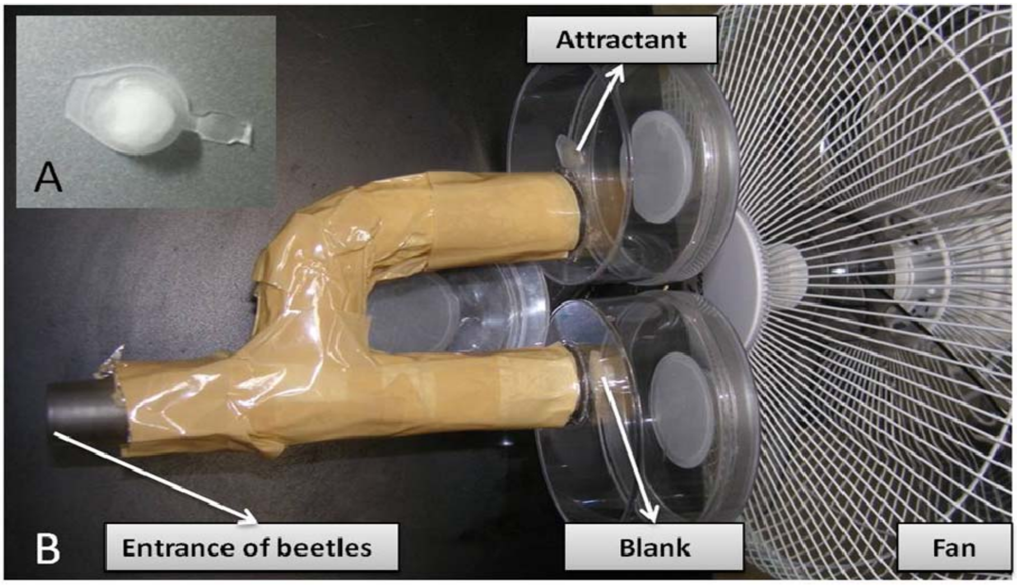

Fig. 1: The device used to evaluate the effectiveness of the tested attractants. A, the attractant container; B, the whole device, which consisted of one y-shaped passage, two collection cages, and a fan.

2.4. Data analysis
All experiments were conducted in three replications and repeated twice, there were different beetles used in the repeated experiment, but the same ones used in the replications. The data are presented as means and standard deviations and any significant differences were determined by one-way analysis of variance using the Duncan multiple comparison module of the Minitab statistical software package, version 13.0 (Minitab Inc., State College, PA, USA). Differences with P values of <0.05 were considered statistically significant.

3. Results
3.1. Diffusion rates and duration of usability of attractants
The diffusion rate and duration of usability of the control (50% ethanol), turpentine oil (with 50% ethanol), α-pinene (with 50% ethanol), and the experimental attractant prepared as described above are shown in Fig. 2. The diffusion rates of turpentine oil (2.10 g/d) and α-Pinene (2.03 g/d) were not significantly different. Both diffused faster than the other two attractants, and 50 ml of either attractant could be effectively used for about 25 days. The diffusion rate of the control (50% ethanol) was moderate (1.34 g/d); it could be used for around 37 days. The experimental attractant diffused slowest (0.97 g/d), which allowed continuous, effective use for about 51 days, representing a time period of usability double that of the positive control.

3.2. Number of beetles in traps
Fig. 3 shows the number of *A. rusticus* beetles trapped per week, along with the weather conditions in Daejeon from 8 August to 5 September in 2013 (minimum and maximum air temperature, precipitation, and air-flow rate information, obtained from the South Korea Meteorological Agency). The first week was characterized by rainfall, high temperature, and little breeze, only 7 beetles were caught this week. In the second week, there was no rainfall, and while a similar temperature and only a slightly higher air flow-rate were seen, substantially more beetles were caught; 13 beetles, almost double the number trapped during the rainy week. The temperature began to decrease during the third week,

culminating in windy days with heavy and continuous rain. During this week, the lowest number of beetles was trapped. Finally, the last week began with rain on the first day, followed by dry conditions, with a further reduction in temperature and stronger wind, the highest number of beetles was caught during this week.

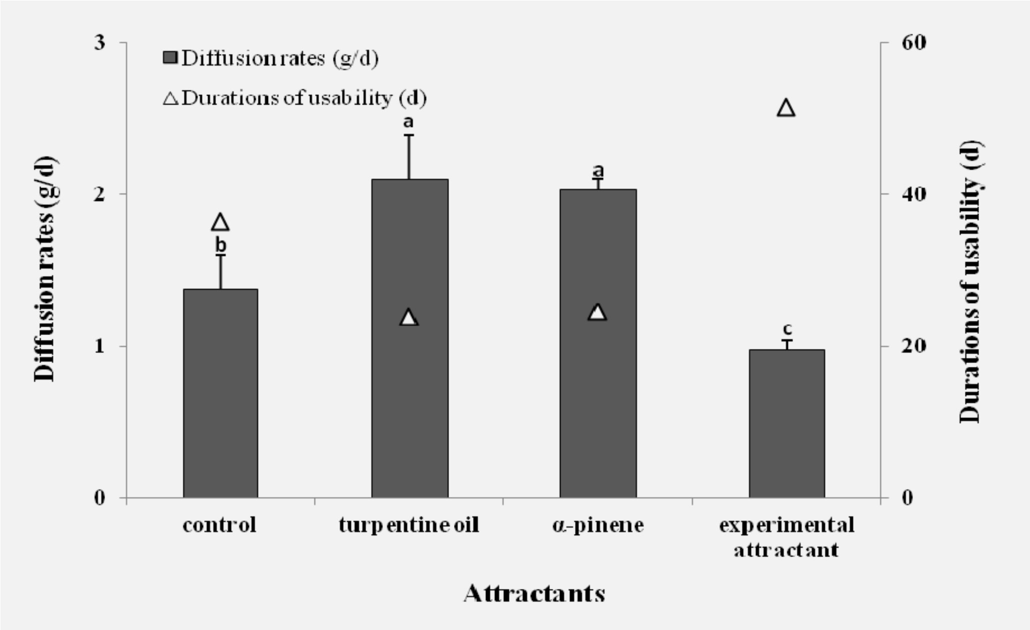

Fig. 2: The diffusion rates and durations of usability of the attractants. Error bars represent the standard deviation of triplicate experiments. Letters indicate differences that are significant at P<0.05 according to the Duncan multiple range test.

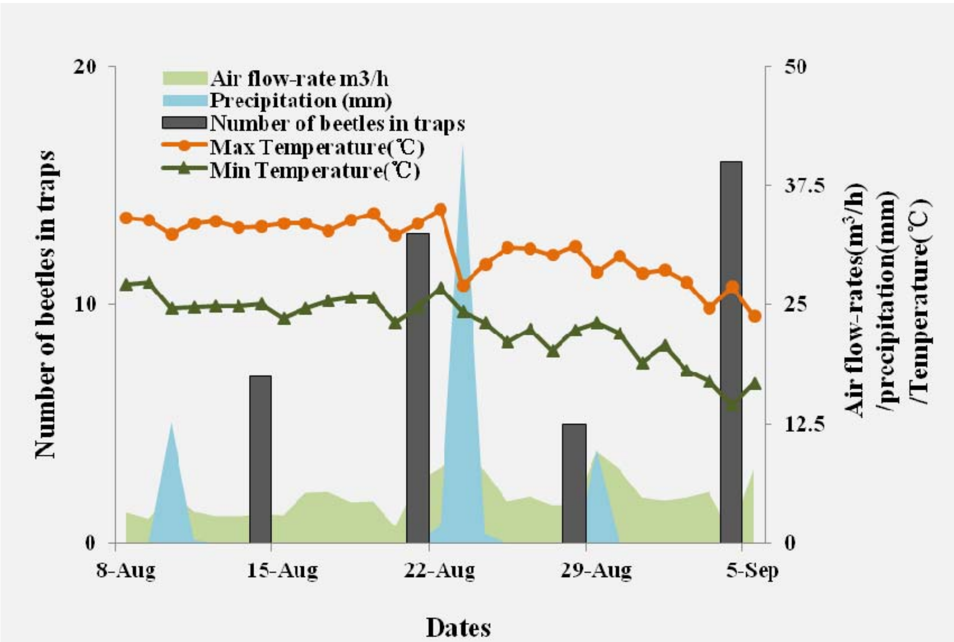

Fig. 3: Number of *Arhopalus rusticus* beetles caught per week in traps, and weather conditions (minimum and maximum air temperature, precipitation, and air-flow rate) in Daejeon during the investigation period.

3.3. Attraction rates of *A. rusticus* by the attractants

The attractive effect of attractants on male and female *A. rusticus* beetles is shown in Fig. 4. Clearly, turpentine oil, one of the positive controls, exerted the greatest attractive effect on both male and female beetles. Turpentine oil was followed by α-pinene, the other positive control, which had a stronger attractive effect on females than on males. The experimental attractant was slightly inferior to the positive control, but its attractive capacity showed no significant difference between males and females. The control, ethanol, performed poorly compared with the other three attractants to *A. rusticus*, and it attracted more males than females.

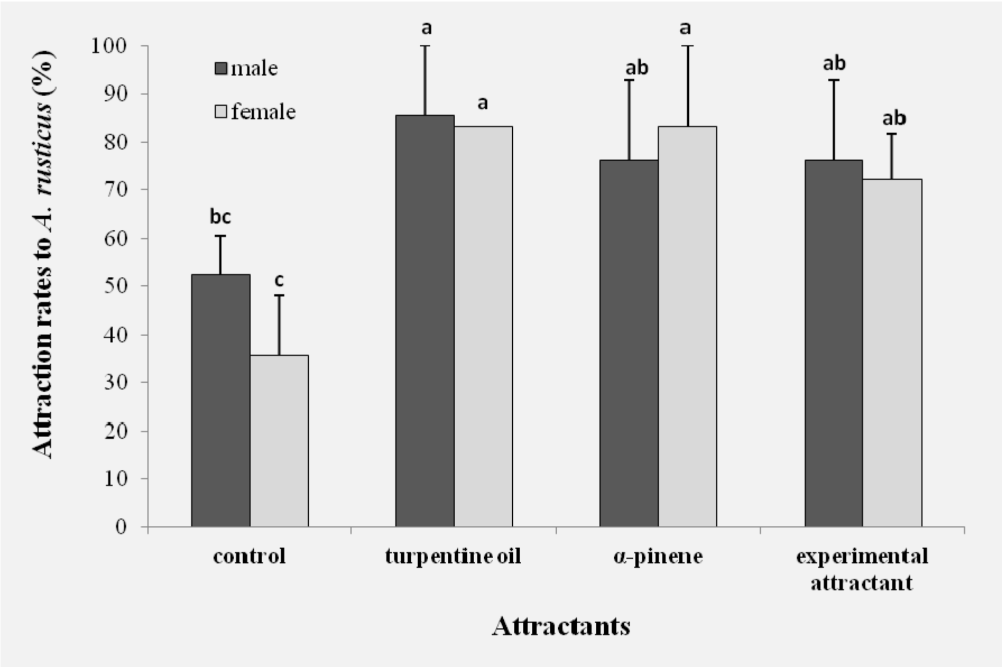

Fig. 4: The attraction rate of the control (50% ethanol), turpentine oil (with 50% ethanol), α-pinene (with 50% ethanol) and experimental attractant. The rate was measured for male and female *A. rusticus*. Error bars represent standard deviations from triplicate determinations. Letters indicate differences that are significant at P<0.05 according to the Duncan multiple range test.

4. Discussion

There is a reverse correlation between the diffusion rate and the duration of usability. It was observed in one report that a faster diffusion rate (300 mg/d) was superior to a slower one (150 mg/d) in terms of the number of beetles caught [14]. However, the experimental attractant we prepared diffused at a rate of about 1,000 mg/d, well above the range examined in this previous report. This diffusion rate is a practical one for use in field traps; diffusion rates much higher than this would indicate very rapidly diffusing attractants with periods of usability that would be too short to efficiently capture sufficient numbers of beetles in the forest (data not shown).

It appears that the attractive ability of the attractant was heavily influenced by the weather conditions. The number of beetles trapped differed each week, and this variance appeared to be related to weather conditions (temperature, rainfall, wind, etc.) as well as the life cycle of *A. rusticus*. While the sampling described here is too limited to draw any conclusions about the effects of weather on the traps, the observed pattern is suggestive. Follow up studies over longer periods of time, and with more traps, will be able to shed more light on this question. At the same time, we are working toward the goal of improving the bait container and trap design to reduce the influence of weather.

In addition, from May to September, the beetle life cycle usually goes through pupation, eclosion, maturation feeding, mating, and oviposition [15]. The present trapping trial was started in May, but in the first three months, very few *A. rusticus* beetles were caught (data not shown). Later, the catch of beetles consisted almost entirely of unmated adults, with no significant difference between the numbers of males and females. When males and females were kept together, they had a strong tendency to mate. This suggests that the bait is more attractive to the virgin adult *A. rusticus* beetle. Some nematodes were dissected from the beetles and from wilting pine, but the nematode species could not be identified.

The small difference between turpentine oil and α-pinene in attraction of females could indicate that the other ingredients (β-pinene, camphene, limonene, β-phellandrene, and myrcene) or the proportion of components in turpentine oil play a role in attraction. The control, ethanol, has been used as an attractant to catch cerambycid, scolytid, and clerid beetles [16],but it performed poorly for attraction to *A. rusticus*. The experimental attractant was slightly inferior to the positive control, but its attractive capacity showed no significant difference between males and females, and this attribute was supported by the field catch results. Considering the lower cost and the longer usage life, the experimental attractant could represent a useful tool for application in the field. And the attracting effect of the attractants evaluated by the evaluation apparatus in vitro showed a credible results, which was expected to facilitate the screening of multiple attractants.

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