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Effect of Time, Temperature, and Root Exudates on the Development of *Rotylenchulus reniformis*

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ABSTRACT

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The young female is the only infective stage in the life cycle of *Rotylenchulus reniformis*. This feature poses interference in the precision of the researches of *R. reniformis* due to the extra effort required to obtain the effective inoculums, the infective stage of the nematodes, after hatching of the eggs. Using the second-stage juveniles as inoculum source has the potential problem of inconsistent level of effective inoculums and the comparison between different researches cannot be made. Understanding of the factors affecting the development of *R. reniformis* will help the improvement of the preparation of effective inoculums for researches. The effect of time, temperature, and root exudates on the development of the reniform nematodes was investigated. The percentage of young females and males increased with time after hatching and reached 26.0% and 17.7%, respectively, at 21 days in water at room temperature. There were very few young females developed when the nematodes were kept at 15°C throughout the experiment. The percentage of young females reached the highest, 40.6%, at 25 days when the nematodes were kept at 28°C throughout the experiment. The percentage of young females was 38.9% at 25 days when the nematodes were kept at 15°C for one week while collecting the newly hatched nematodes and then kept at 28°C throughout the experiment. The amount of males was the lowest at 15°C among the temperature treatments. The percentages of males increased with time in the 15°C-28°C and 28°C treatments and reached 40% and 36.8%, respectively, at 25 days. The percentages of young females and males in the root exudates of mung bean were not significantly higher than that in the control. The mean percentages of the males were higher than that of the females in the 15°C treatment. The male to female ratio was close to 1:1 from 16 to 25 days in the 15°C-28°C and 28°C treatments. The males developed faster than the young females at low temperature. The results of our study may help in the preparation of the infective stage of *R. reniformis* and the amount of effective inoculums used in each experiment can then be pinpointed to facilitate the comparison of researches between different researchers. Besides helping in the improvement of the precision of *R. reniformis* researches, our study also adds to the understanding of the basic biology of this nematode.

Keywords: development, exudates, infective stage, inoculums, reniform nematode, *Rotylenchulus*, sex ratio, temperature, time

INTRODUCTION

The reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, 1940, is an important plant-parasitic nematode in tropical and subtropical areas of the world. It affects many economically important crops [17]. Robinson [15] reported that *R. reniformis* has replaced the root-knot

nematode, *Meloidogyne incognita*, as the major nematode of cotton in Mississippi, Louisiana, and Alabama and caused an estimated $130M annual loss to the U.S. cotton crop. The life cycle of the reniform nematode has been described by Linford and Oliveira [11]. It is a semi-endoparasite with the young female as its infective stage.

Many of the plant-parasitic nematodes, such as the root-knot nematode and the cyst nematode, invade host plants with their second-stage juveniles which begin to develop after feeding on the hosts [20, 28]. The second-stage juveniles of the migratory endoparasites, such as the lesion nematode and the burrowing nematode, also begin to develop into other stages of their life cycles after feeding on the hosts [18, 29]. The infective stage of *R. reniformis*, the young females, develops from the second-stage juveniles without feeding [11]. This unique feature poses interference in the precision of the researches of *R. reniformis* due to the extra effort required to obtain the effective inoculums, the infective stage of the nematodes, after hatching of the eggs. Using the second-stage juveniles of *R. reniformis* as inoculum source has the potential problem of inconsistent level of effective inoculums because the second-stage juveniles may not all develop into young females or the percentage of development may vary among different experiments. Understanding of the factors affecting the development of *R. reniformis* will help the improvement of the preparation of effective inoculums for researches. Our objective was to study the effect of time, temperature, and root exudates on the development of the reniform nematode. As *R. reniformis* reproduces mainly by amphimixis [17, 21], the development of males was included in the study besides young females.

MATERIALS AND METHODS

Preparation of nematodes

A population of *R. reniformis* was originally collected from the experimental station of the National Taiwan University and cultured on mung bean (*Vigna radiata* (L.) Wilczek). Egg masses were dissected from roots and hatched in a hatching chamber [24]. Fresh second-stage juveniles were collected every day and kept at 15°C or 28°C, depending on the experiment.

Experiment 1 - Effect of time on nematode development

Fresh second-stage juveniles collected from the same day were used in the experiment. Each replicate consisted of 200 nematodes in distilled water in a Petri dish (5.4 cm diam.). Parallel lines approximately 5 mm apart were drawn at the bottom of the Petri dish prior to loading the nematodes to facilitate counting. The nematodes were kept at room temperature (20-25°C). The number of young females and males were counted at 0, 3, 6, 9, 12, 15, 18, and 21 days under a dissecting microscope (SZH-ILLB, Olympus Optical Co., Ltd., Tokyo). After each counting, the Petri dish was covered with lid and resealed with Parafilm to avoid drying. There were four replicates. The experiment was repeated twice. The % nematodes was calculated based on the number of young females or males divided by the total number of nematodes per dish.

Experiment 2 - Effect of temperature on nematode development

The first set of freshly hatched second-stage juveniles was collected every day for one week and kept at 28°C throughout the experiment (Treatment 28°C-28°C). The second set was kept at 15°C for one week while collecting the newly hatched nematodes and then kept at 28°C throughout the experiment (Treatment 15°C-28°C). The third set was kept at 15°C throughout the experiment (Treatment 15°C-15°C). The temperature of 15°C was chosen because it was the temperature used to store live nematodes to preserve their freshness [26] as a routine practice in our laboratory. The temperature of 28°C was chosen because both mung bean and the nematodes grew well at 28°C in our preliminary studies. The treatment 15°C-28°C was designed to preserve the freshness of the nematodes at 15°C while waiting to cumulate sufficient amount of nematodes and then allows good development of the nematodes at 28°C. Each treatment had four replicates. Each replicate consisted of 100 nematodes in distilled water in a Petri dish (5.4 cm diam.). Parallel lines approximately 5 mm apart were drawn at the

bottom of the Petri dish prior to loading the nematodes to facilitate counting. The number of young females and males were counted at 7, 10, 13, 16, 19, 22, and 25 days under a dissecting microscope (SZH-ILLB, Olympus Optical Co., Ltd., Tokyo). After each counting, the Petri dish was covered with lid and resealed with Parafilm to avoid drying. The experiment was repeated twice. The % young females was calculated based on the number of young females divided by the total number of nematodes per dish. The % males was calculated based on the number of males divided by the total number of nematodes per dish.

Experiment 3 - Effect of root exudates on nematode development

Freshly hatched second-stage juveniles were collected every day for one week and kept at 28°C throughout the experiment. Root exudates were collected from 1-week old mung bean seedlings grown in culture pouches with recycled paper as the supporting substrate for the seedlings [25]. The exudates collected from different pouches were mixed in a flask and the aliquot of 3 ml of the mixture was pipetted into a Petri dish (5.4 cm diam.) and mixed with 1 ml of nematode suspension containing 200 nematodes. Parallel lines approximately 5 mm apart were drawn at the bottom of the Petri dish prior to loading the nematodes to facilitate counting. Distilled water was used in place of the exudates as the control. The number of young females and males were counted at 7, 10, 13, 16, 19, and 22 days after the treatment under a dissecting microscope (SZH-ILLB, Olympus Optical Co., Ltd., Tokyo). After each counting, the Petri dish was covered with lid and resealed with Parafilm to avoid drying. There were four replicates. The experiment was repeated twice. The % young females was calculated based on the number of young females divided by the total number of nematodes per dish. The % males was calculated based on the number of males divided by the total number of nematodes per dish.

Arcsine square root transformation was performed for percentages of all the above experiments before statistical analysis. One-way analysis of variance was carried out with SAS 9.1 software (SAS Institute, Cary, NC, USA) and treatment means were compared with Duncan's multiple range test or Student's t-test at the 95% level of confidence. In the repeated experiments, the data were combined with the first one because the same trend was observed in the two experiments.

RESULTS

In Experiment 1, the percentage of young females and males increased with time and reached 26.0% and 17.7% (mean of four replicates), respectively, at 21 days in water after hatching (Fig. 1). There was no significant difference between young females and males according to t-test.

No females were found at 7 days in the 15°C-28°C and 28°C-28°C treatments in Experiment 2. The male to female ratio was close to 1:1 from 16 to 25 days in the 15°C-28°C and 28°C-28°C treatments (Table 1). The highest male to female ratio was 6.1:1 which occurred at 10 days in the 15°C-15°C treatment. However, the highest male to female ratio at the last sampling date was 2.6:1 which occurred at 25 days in the 15°C-15°C treatment (Table 1).

There were very few young females developed in the 15°C-15°C treatment (Fig. 2). The percentages of young females in the 15°C-28°C and 28°C-28°C treatments were similar. They both reached the highest at 25 days, 38.9% and 40.6%, respectively. The amount of males was the lowest at 15°C-15°C among the three temperature treatments (Fig. 3). The percentages increased with time in the 15°C-28°C and 28°C-28°C treatments and reached 40% and 36.8%, respectively, at 25 days.

The percentages of young females in the root exudates of mung bean were slightly higher than that in the control (Fig. 4). However, there was no significant difference according to t-test. The effect of the root exudates on the development of the males (Fig. 5) was similar to that of the young females.

DISCUSSION

The appearance of young females and males at day 3 in

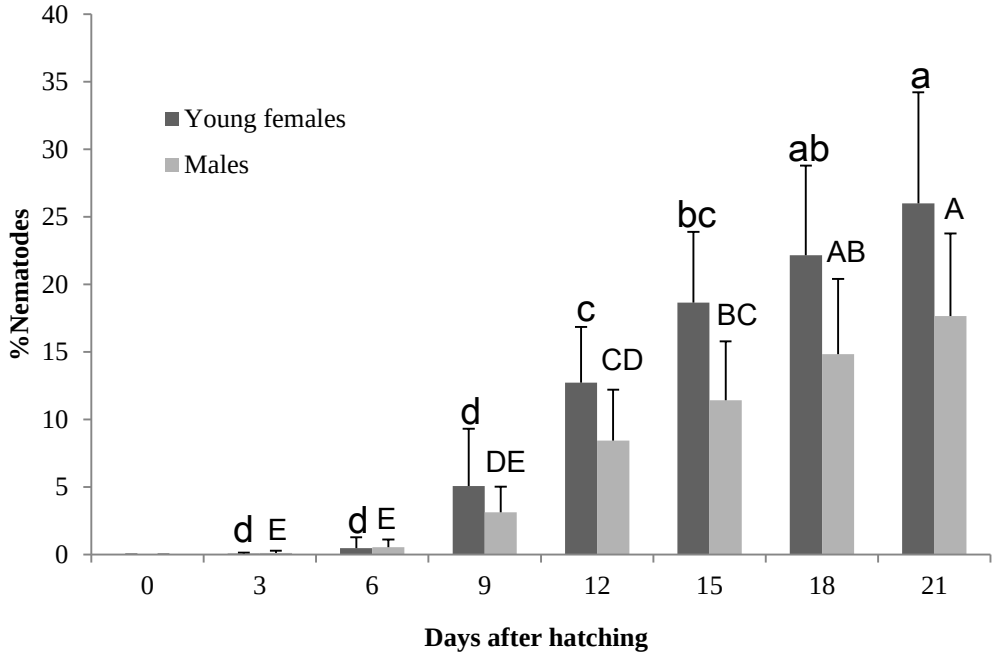

Fig. 1. The development of young females and males of *Rotylenchulus reniformis.* The % nematodes was calculated based on the number of young females or males divided by the total number of nematodes per dish. Bars of young females or males with different letters are significantly different at $P \leqq 0.05$ according to Duncan's multiple range test after arcsine square root transformation. Each bar represents the mean of non-transformed data of four replicates ± standard deviation.

Table 1. The sex ratio of *R. reniformis*[x] (Experiment 2)

	Male/female		
Days	15℃-15℃[y]	15℃-28℃	28℃-28℃
7	2.0 (0.45/0.23)[z]	0.2:0 (0.23/0)	0.6:0 (0.625/0)
10	6.1 (1.4/0.23)	0.5:0 (0.45/0)	2.4±1.7 (10.95/4.63)
13	2.0 (1.85/0.93)	1.5±1.0 (13.15/8.98)	1.2±0.5 (20.78/17.73)
16	1.6 (1.85/1.18)	1.0±0.3 (24.75/25.05)	1.0±0.2 (30.68/30.2)
19	1.8 (2.08/1.18)	1.0±0.1 (29.68/30.88)	1.0±0.2 (33.88/33.05)
22	2.2 (2.55/1.18)	0.9±0.1 (34.4/37.33)	0.9±0.3 (35.35/37.68)
25	2.6 (4.88/1.88)	1.0±0.1 (40/38.88)	0.9±0.3 (36.78/40.55)

[x] Mean of four replicates ± standard deviation.

[y] Standard deviation cannot be calculated due to absence of females in some replicates.

[z] The male/female ratio (number of males/number of females).

Experiment 1 (Fig. 1) is sooner than that reported by Bird [(1)]. Bird observed eight individuals developed from J2 into males in 10 to 19 days and four individuals developed from J2 into young females in 13 to 16 days at 24℃. Although following the development of individual nematode can provide accurate timing of the development, it has the drawback of being limited in sample size due to its laborious nature of the work. It remains possible that some juveniles may develop faster than he has observed. However, the possibility also exists that a few eggs might have hatched before the egg masses were picked and those juveniles from the early-matured eggs were transferred along with the egg

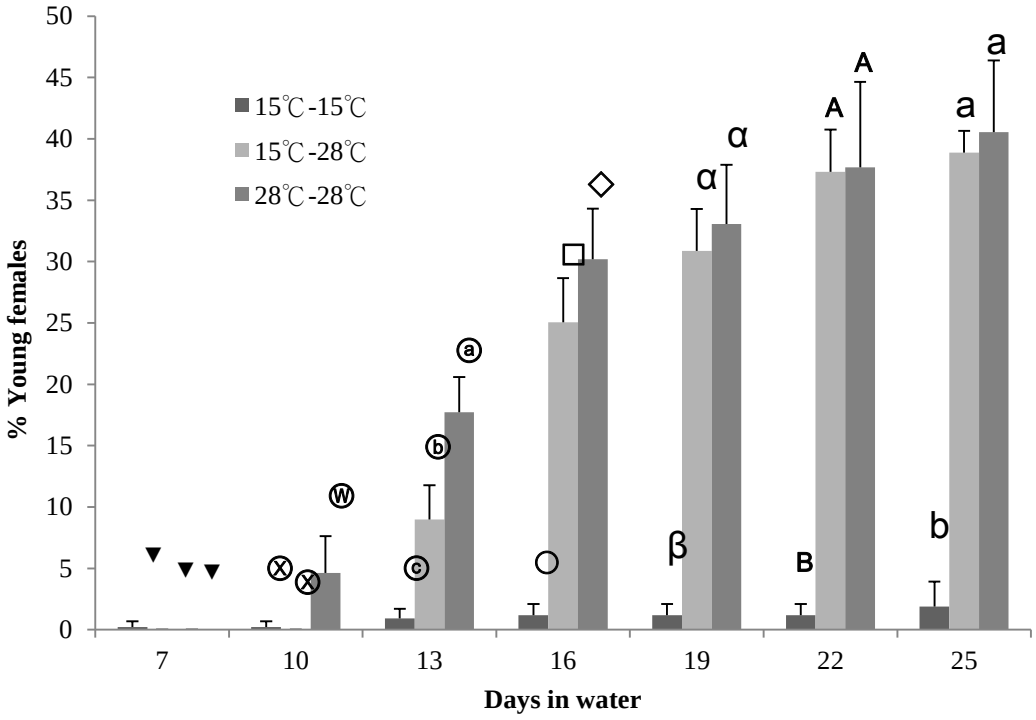

Fig. 2. The effect of temperature on the development of young females of *Rotylenchulus reniformis*. Bars at each sampling date with different symbols or letters are significantly different at $P \leqq 0.05$ according to Duncan's multiple range test after arcsine square root transformation. Each bar represents the mean of non-transformed data of four replicates ± standard deviation.

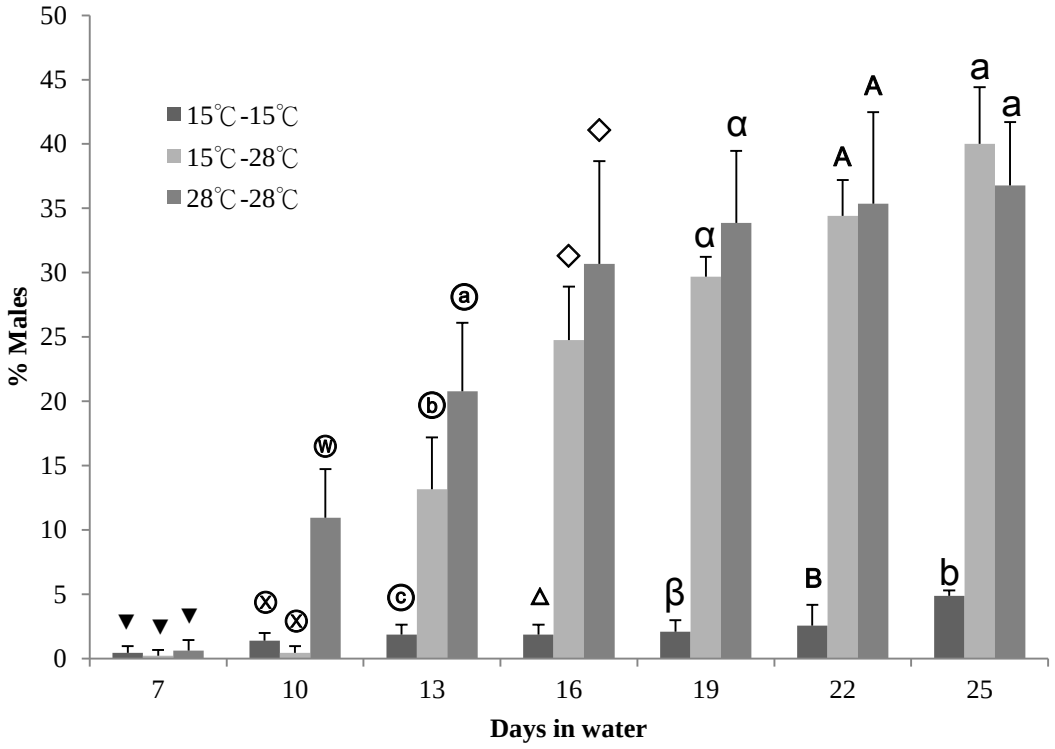

Fig. 3. The effect of temperature on the development of males of *Rotylenchulus reniformis*. Bars at each sampling date with different symbols or letters are significantly different at $P \leqq 0.05$ according to Duncan's multiple range test after arcsine square root transformation. Each bar represents the mean of non-transformed data of four replicates ± standard deviation.

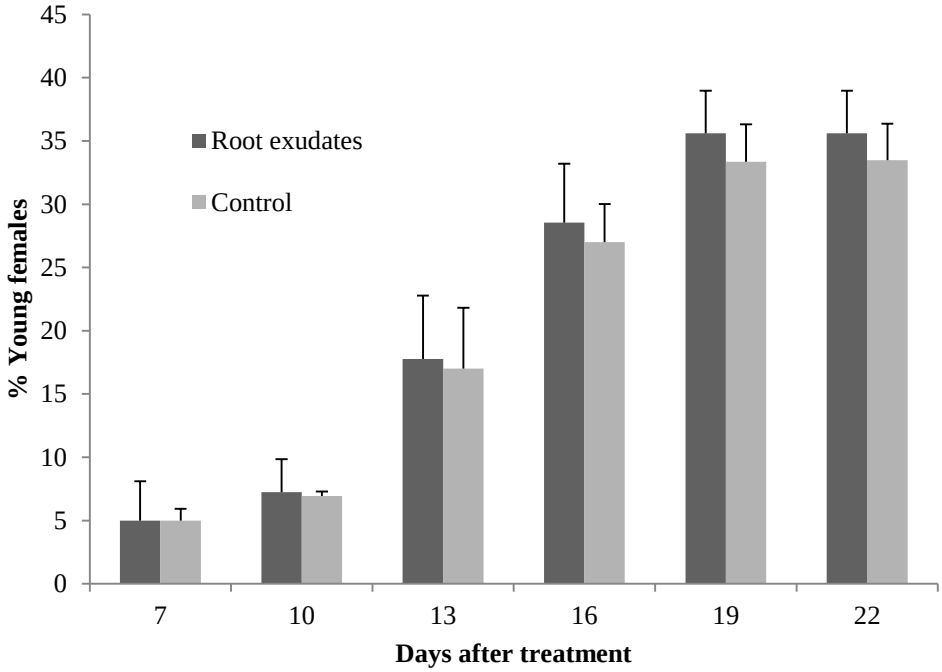

Fig. 4. The effect of root exudates on the development of young females of *Rotylenchulus reniformis*. There was no significant difference between root exudates and the control at each sampling date according to Student's t-test.

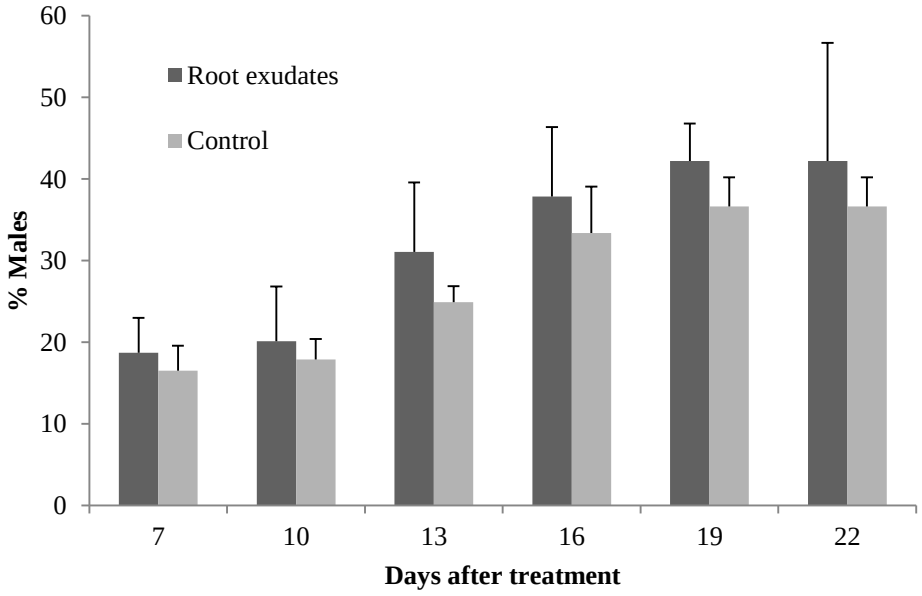

Fig. 5. The effect of root exudates on the development of males of *Rotylenchulus reniformis*. There was no significant difference between root exudates and the control at each sampling date according to Student's t-test.

masses and caused the early appearance of the young females and males in our experiment. The male to female ratios were close to 1:1 from 16 to 25 days in the 15˚C-28˚C and 28˚C-28 ˚C treatments (Table 1). In the control of Experiment 3, in which the temperature was equivalent to the 28˚C-28˚C treatment in Experiment 2 and therefore can serve as a repeat of that part of the experiment, the male to female ratios were similar to that of 28˚C-28˚C treatments in Experiment 2 at 10 to 22 days. The male to female ratios in the 15˚C-15˚C treatment, ranging from 1.6 to 6.1, were consistently higher than that in the 15˚C-28˚C and 28˚C-28˚C treatments at every sampling date. It is apparent that low temperature favors the

development of males of *R. reniformis*. This is in agreement with the findings on *Meloidogyne incognita* by Davide and Triantaphyllou [4]. They found more males of *M. incognita* at 15° C than 20°, 25°, 30° or 35℃. Influence of environment on nematode sex differentiation has been a subject of interest among researchers in the past years and an extensive review was done by Triantaphyllou [22]. Besides plant-parasitic nematodes, environmental effect on sex ratio has also been reported for *Drosophila* [3], fish [2], reptiles [9, 27], and birds [7].

The higher male to female ratios at the earlier sampling times in Experiment 2 indicated that males probably developed sooner than the females at the beginning and then females started to catch up and caused the ratios to drop later. This is in agreement with the report of Sivakumar and Seshadri [19]. They found that the female of *R. reniformis* can complete its life cycle in 24-29 days and the male in 16-20 days.

The percentage of males at 22 days was 34.4% in the 15 ˚C-28˚C treatment, 35.4% in the 28˚C-28˚C treatment, and 36.7% in Experiment 3 (equivalent to 28˚C-28˚C treatment) (Figs. 3 & 5). These percentages are very close to each other indicating that the rate of development of males at 28˚C is very stable.

In Experiment 1, maximum amount of young females were obtained at 21 days after hatching in water (Fig. 1). The highest percentage of young females, 40.55%, appeared in the treatment of 28˚C-28˚Cat 25 days after hatching in water (Fig. 2). Although it might produce more young females beyond 25 days, those developed earlier might have become too old and reduced their infectivity.

The effect of temperature on the development of males (Fig. 3) was similar to that of the young females (Fig. 2). The temperature of 15˚C hiders the development of young females and males of *R. reniformis* (Figs. 2 & 3), even though it is good for preserving the freshness of nematodes [26]. The percentages of young females in the treatment of 15˚ C-28˚C were similar to that of 28˚C-28˚C (Fig. 2). It may be necessary to cumulate nematodes hatched for more than one day to obtain sufficient amount of inoculums for some experiments. In this case, the 15˚C-28˚C treatment would be a better choice since it combines the advantages of both 15˚C and 28˚C, *i.e*., it preserves the freshness of the nematodes at 15˚C while waiting to cumulate sufficient amount and then allows good development of the nematodes at 28˚C. In order to obtain optimum effective inoculums of *R. reniformis*, a balance must be kept between freshness of nematodes and maximum amount of young females. Although no research has been done regarding to the temporal effect on the reduction of infectivity of *R. reniformis*, it was reported that infectivity of second-stage juveniles of *Meloidogyne incognita* was reduced through aging [23]. Further research on the effect of aging on the infectivity of *R. reniformis* will help in determining how long one should wait to obtain maximum amount of young females without sacrificing their infectivity.

Prior to infecting the host roots, the reniform nematodes are in contact with root exudates in the rhizosphere. Root exudates have been reported to interact with plant-parasitic nematodes, such as stimulating the hatching of cyst nematodes [6, 30], and repelling the root-knot nematode juveniles [5]. There have been no reports on *R. reniformis* regarding the effect of root exudates. The result of Experiment 3 showed that root exudates of mung bean did not significantly increase the percentages of young females and males (Figs. 4 & 5). The constituents of root exudates may vary among different plant species. Therefore, the effect of root exudates from other host plants cannot be generalized based on this study.

Since young female is the only infective stage of the reniform nematode, it is necessary to know how many young females are used for inoculation. Rebois[14] used 300 nematodes (males, larvae and young females) with the ratio of males to females approximately 1:1 as the inoculum. However, the percentage of the young females in the inoculum was not given. Therefore, exactly how many young females were used for inoculation was unknown. Robbins and Rakes [13] inoculated the soybean seedling with 1000 vermiform reniform nematodes without any information of the sex ratio or the percentage of the young females. Parkhi, *et al*. [12] inoculated the cotton plant with 6,000 motile nematodes also without any information on the young females. Comparisons between different researches cannot

be made without the information on the amount of effective inoculums. The results of our study may help in improving the precision of experiments on *R. reniformis* by pinpointing the amount of effective inoculum used in each experiment to facilitate the comparison of researches between different researchers.

Temperature has been reported to affect various aspects of the nematodes, such as survival [26], egg hatching [16], and infectivity [14]. The effects of temperature on the life cycle and embryogenesis of the reniform nematode have been studied [10, 14]. However, researches on the effect of temperature on the development of the reniform nematode from juveniles to males and young females are lacking. Our study adds to the understanding of the basic biology of this nematode. Understanding of the biology of nematodes may contribute to the development of environment-friendly strategies for nematode control in the future. In addition, it may contribute to other aspects. For example, the researches on the development of the nematode *Caenorhabditis elegans* led to the discovery of genetic regulation of organ development and programmed cell death [8] which contributed immensely to the cancer researches and the researchers were awarded with the Nobel prize in 2002. The development of the juveniles of *R. reniformis* into young females without feeding is a unique feature, the study of which encompasses the potential of contributing to the researches of other organisms.

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摘 要

蔡碧雲[1,2]、黃慧真[1]. 2011. 時間、溫度、及根部分泌物對腎形線蟲發育之影響. 植病會刊 20: 108-117. ([1]台北市 國立台灣大學植物病理與微生物學系；[2]聯絡作者，電子郵件：bieyntm@ntu.edu.tw；傳真：+886-02-23636490)

腎形線蟲 *Rotylenchulus reniformis* 的年輕雌蟲是侵入寄主的唯一齡期。這個特性對 *R. reniformis* 之研究的精確度造成干擾，因為卵孵化後尚需額外的努力才能得到具侵入能力的齡期。用二齡幼蟲接種可能造成有效接種原之量不一致的問題，使得不同實驗之間無法做比較。了解影響 *R. reniformis* 發育的因子有助於製備研究所需之有效接種原。本研究包括時間、溫度、及根部分泌物對腎形線蟲發育之影響。年輕雌蟲及雄蟲之百分比隨著時間而增加，於室溫下在水中 21 天後分別達到 26.0%及 17.7%。當線蟲一直保存在 15˚C 時僅出現極少數之年輕雌蟲。當線蟲一直保存在 28˚C 時年輕雌蟲之百分比在 25 天後達到最高，40.6%。在收集新孵化之線蟲時先保存在 15˚C 一星期然後才以 28˚C 處理者年輕雌蟲之百分比在 25 天後達到 38.9%。雄蟲之數目在 15˚C 之處理最低。在 15˚C-28˚C 及 28˚C 之處理雄蟲之百分比隨時間而增加，於 25 天後分別達到 40%及 36.8%。綠豆根部分泌物處理者年輕雌蟲及雄蟲之百分比與對照組無顯著差異。在 15˚C 之處理雄蟲之百分比高於年輕雌蟲。在 15˚C-28˚C 及 28˚C 之處理於 16 至 25 天之間雄雌比率接近 1:1。低溫時雄蟲之發育較年輕雌蟲快。本研究之結果有助於製備 *R. reniformis* 具侵入能力之齡期，而每個實驗可因而精確指出所使用之有效接種原的量，有利於不同實驗之間的比較。本研究之結果亦對腎形線蟲之基本生物學之瞭解有所貢獻。

關鍵詞：發育、根部分泌物、侵入齡期、接種原、腎形線蟲、性別比率、溫度、時間、*Rotylenchulus*