PIPER LONGUM L—A NEW HOST RECORD FOR *COLLECTOTRICHUM GLOEOSPORIOIDES* PENZ

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PIPER LONGUM L the Indian long pepper is a slender aromatic climber with perennial woody roots occurring in the ever green forests of western ghats from Konkan to Travancore. The fruits, roots and thicker parts of stem are cut and dried and used as an important drug in the Ayurvedic and Unani systems, mainly for diseases of respiratory tract.

During March 1982, a severe leaf spot disease of *P. longum* was noticed at Regional Agricultural Research Station, Pilicode. The symptoms were mainly confined to the leaves and tender twigs. Infection on the leaves generally originated as discoloured areas at the tip or occasionally near the margin (figure 1). In severe cases of infection the leaves are shrivelled and dried. The causal organism was isolated in potato dextross agar medium and the pathogenicity was established by spraying the spore suspension of conidia from 10 days old culture. The causal organism was identified as *Collectotrichum gloeosporioides* Penz. A review of literature revealed that there is no record of *C. gloeosporioides* Penz. causing leaf spot on long pepper.

Morphological studies of the fungus revealed that the hyphae are hyaline, septate and branched. The conidia are produced on acervuli and are hyaline, formed on single conidiophores. Conidia are cylindrical or oblong, one celled and measures 10.24–16.65 μ long and 3.14–4.50 μ broad.

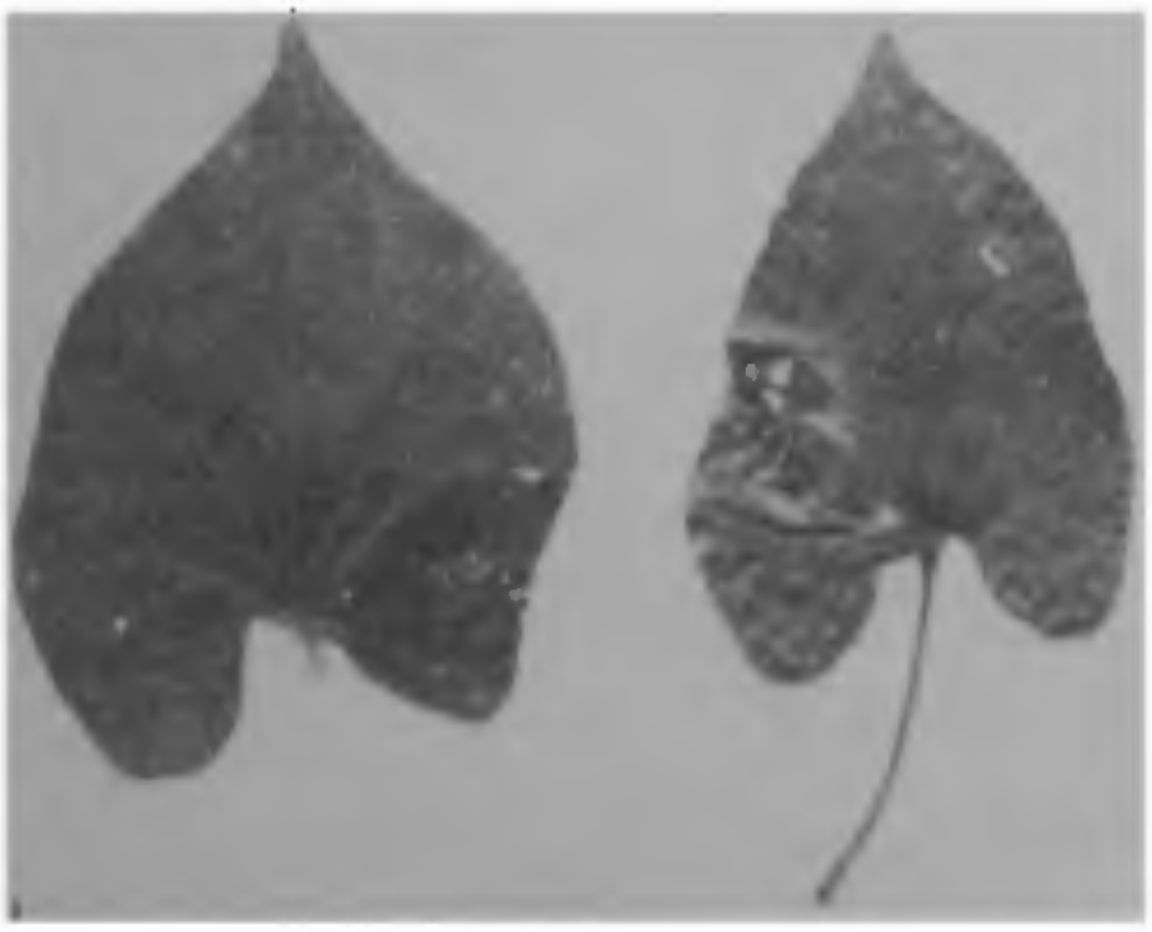

Figure 1. Diseased leaves showing symptoms.

It was found that the pathogen could infect the leaves of pepper (*P. nigrum*) and produce similar symptoms, when inoculated in the field.

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ISOLATION AND CHARACTERIZATION OF ASSIMILATORY NITRATE REDUCTASE FROM *AZOSPIRILLUM BRASILENSE*

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NITRATE reductase of both assimilatory and dissimilatory types are reported to occur in *Azospirillum brasilense*[1]. Assimilatory nitrate reductase (NR) occurs only under aerobic conditions as it reduces NO_3^- to NO_2^- which is further reduced to ammonia by nitrite reductase before being finally used up by cells. Dissimilatory nitrate reductase on the other hand occurs under anaerobic condition and it catalyses the reduction of NO_3^- to nitrite which is further reduced to N_2 by several enzymes with the production of ATP. The assimilatory nitrate reductase in algae and higher plants has been thoroughly characterized and it requires NADH as the electron donor[2,3]. Assimilatory nitrate reductase from *Azotobacter chrococcum*[4] has been isolated and characterized. The isolation and biochemical characterization of the assimilatory nitrate reductase from *A. brasilense* is described here.

The culture *A. brasilense* (ATCC 29145, Strain No. 7) obtained from J. Dobereiner[5] was maintained on nutrient agar. The inoculum was prepared by growing cells in glutamate medium with aeration at 35° C for 20 hr. The nitrogen-free medium[6] was used with slight modifications (in g l of distilled water) as follows: K_2HPO_4, 6.0; KH_2PO_4, 4.0; NaCl, 0.1; $MnSO_4.2H_2O$, 0.01; $Na_2MoO_4.2H_2O$, 0.02; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; Malic acid, 5.0; KOH, 4.5 with pH adjusted to 7.0. The glutamate and nitrate growth media were prepared by adding 10 g of K-glutamate and 2.02 g of KNO_3 in 1000 ml of nitrogen-free medium respectively.

The bacteria were harvested by centrifugation at 6000 x g for 10 min and washed with 0.1M phosphate buffer, (pH 7.5). The washed pellets resuspended in cold buffer supplemented with 1 mM EDTA and 1mM cysteine were subjected to 300 watts ultrasonic probe (Braun Sonic -1510) until the turbidity measured as extinction at 660 nm decreased by at least 90%. The sonicated crude extract was centrifuged at 30,000 x g for 30 min at 4° C and the supernatant was subjected to ammonium sulphate fractionation. Protein fraction precipitating between 30–55% saturation was collected and dissolved in 3ml of 0.1M phosphate buffer (pH 7.5), and used for further studies after overnight dialysis at 5°C against 0.01 M phosphate buffer (pH 7.5). No nitrite reductase activity was found under these conditions. NO_2^- concentration in culture filtrates was determined following centrifugation of cells at 6000 x g for 10 min. To 0.1 ml of culture filtrate, 2.0 ml of 1:1 (v/v) freshly-mixed solution of 0.02% N-1-naphthylethylene diamine dihydrochloride and 1% sulphanilamide in 1.5 M HCl were added. After 15 min the reading was taken at 540 nm with Beckman Spectrophotometer (Model 24).

Proteins were determined using Lowry's method[7] and NR enzyme assay was done using the technique of Lowe & Evans[8]. One unit of enzyme activity here has been defined as the amount of enzyme producing 1 nano mole NO_2^-/min under the conditions employed. The specific activity here is expressed as enzyme unit/mg of protein.

Figure 1 shows that NO_3^- supported the aerobic growth of *A. brasilense* in liquid medium when it was used as the sole N-source. Among the three concentrations tried (10, 20 & 30 mM), best growth was obtained with 20 mM NO_3^-. Cells were also grown in liquid basal medium with KNO_2 as the sole nitrogen source. The maximum growth was obtained after 24 hours (O.D. increased from 0.05 to 0.85) which also indicates the assimilatory nature of nitrite reductase.

Cells grown in minimal glutamate medium did not possess any NR activity but the activity was restored after the addition of NO_3^- to the cultures resulting in the accumulation of NO_2^- in the medium. The assimilatory nature of NO_2^- reduction on the other hand was demonstrated by the consumption of added NO_2^- to the medium (figure 2). $(NH_4)_2SO_4$ which did not inhibit the synthesis of assimilatory NR it inhibited the synthesis of assimilatory nitrite reductase. However, chloramphenicol (50 μg/ml) inhibited both the enzymes.

Very high NR activity in cell-free extracts was obtained with NO_3^- as the sole N-source while, the

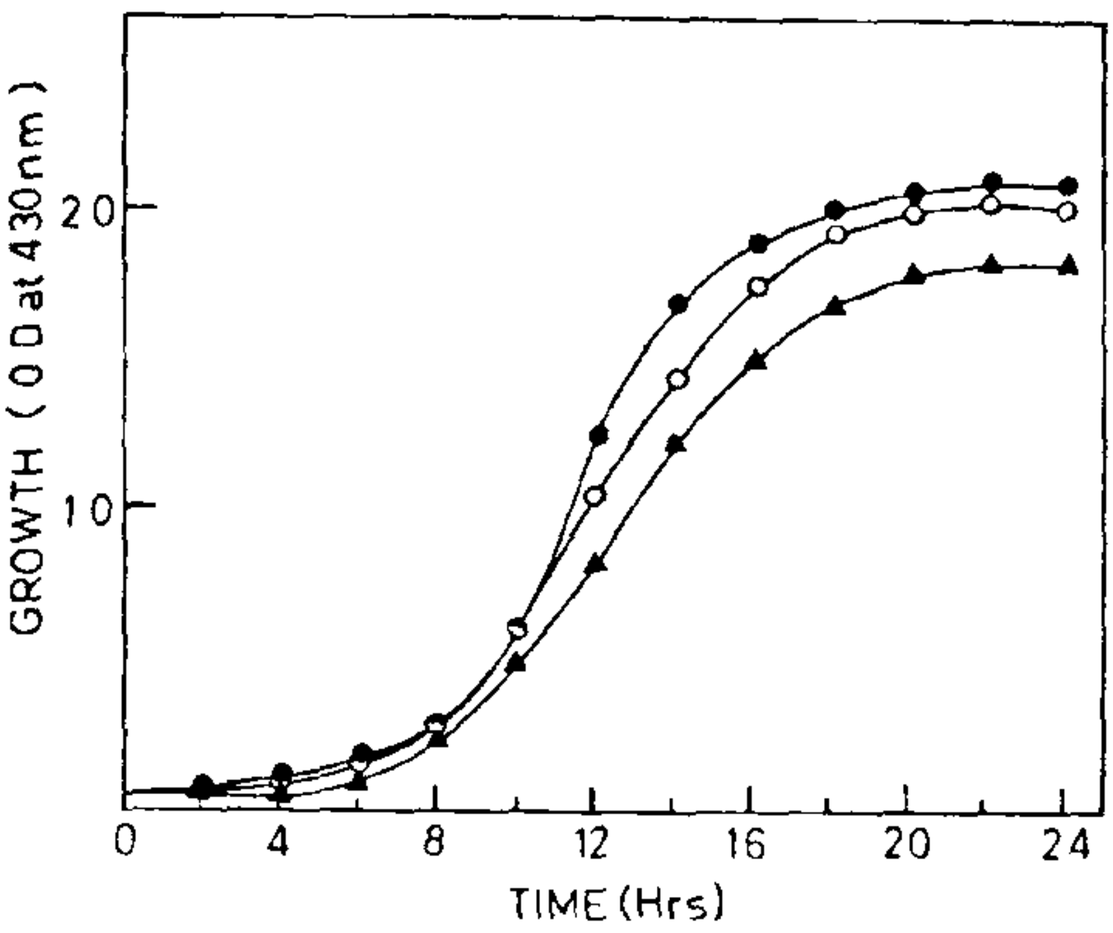

Figure 1. Growth of *A. brasilense* in minimal media having different concentration of nitrate. Symbols: ○—○ 10 mM; ●—● 20 mM; ▲—▲ 30 mM.

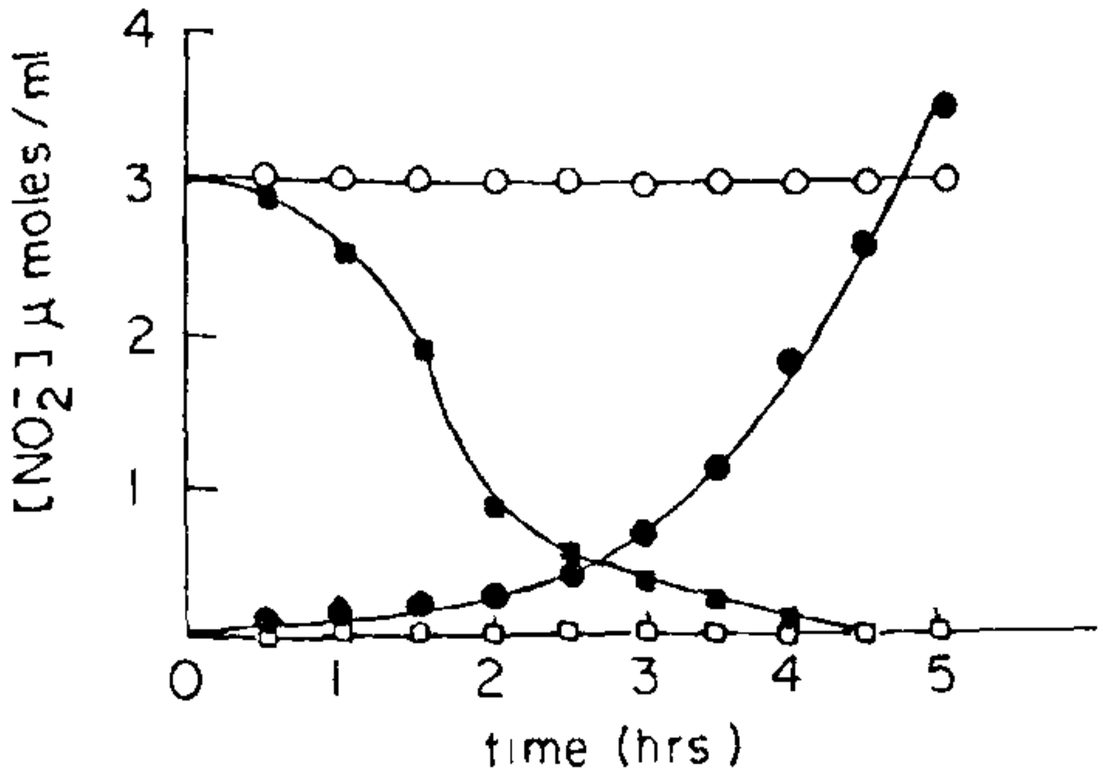

Figure 2. Demonstration of inducible nature of assimilatory nitrate and nitrite reductase in *A. brasilense*. Cells after growing for 18 hours at 35° C with aeration, were divided into different subcultures and incubated further. Samples were removed at different times and nitrite was determined immediately as described in methods. The following compounds were added at the time indicated by arrows:
10 mM KNO_3 or 10 mM KNO_3 + 10 mM $(NH_4)_2SO_4$ (●—●), 10 mM KNO_3 + 50 μg/ml Chloramphenicol (□—□), 3 mM KNO_2 (■—■), 3 mM KNO_2 + 50 μg/ml Chloramphenicol or 10 mM $(NH_4)_2SO_4$ (○—○).

activity was poor with $(NH_4)_2SO_4$ or NH_4NO_3 and no activity was recorded when glutamate was used as the sole N-source. Increased concentrations of

Table 1 *Effect of different nitrogen sources on the cellular level of nitrate reductase in A. brasilense.*

Source of nitrogen	Nitrate reductase nmol/min/mg protein
Glutamate (50 mM)	0.0
$(NH_4)_2SO_4$ (20 mM)	4.5
NH_4NO_3 (20 mM)	12.0
KNO_3 (20 mM)	40.2

Table 2 *Effect of tungstate concentrations on nitrate reductase activity*

Tungstate concentration (μM)	(NO_2^-) nmol/min/mg protein
0	54
100	44
200	32
400	25
800	12

tungstate (> 75 μm) inhibited the aerobic growth of *A. brasilense* on NO_3^-, however, the inhibition could be bypassed by supplying ammonia or NO_2^-.

The NR enzyme from *A.brasilense* has been characterized. Its optimum temperature and pH were recorded as 40°C and 7 respectively. The enzyme lost 75% of its activity following reduction with methylviologen or $Na_2S_2O_4$ but it was resistant to atmospheric oxidation. The enzyme could neither use NADPH or NADH as electron donor. Reduced methylviologen was found to be the most effective electron donor for the reduction of NO_3^- to NO_2^- by NR and the reaction was linear upto 3 min.

Except EDTA, low concentration (1 mM) of metal chelating agents (*e.g.* azide, KSCN or KCNO) stimulated the activity of NR but higher concentrations (> 2 mM) were inhibitory. Low concentration (1 mM) of para-hydroxymercuribenzoic acid (PHMB) and iodoacetamide inhibited the enzyme activity by 25% and 10% respectively. Cysteine could reverse the effect of PHMB but not that of iodoacetamide. The addition of thiol groups stimulated the activity of the enzyme by 16–23%.

Utilization of NO_3^- as the sole N-source, accumulation of NO_2^- in growth medium and, the consumption of NO_2^- by cells indicate that the NR enzyme systems (*i.e.* nitrate reductase and nitrite reductase) of this bacterium are of assimilatory type. Although the existence of assimilatory nitrate reductase in *A. brasilense* has been described by Neyra and Berkum but they did not characterize this enzyme. Most of the biochemical properties of assimilatory NR that have been described here (*e.g.* regulation, repression and optimum conditions of enzyme activity etc) corroborate most in details with that reported in other systems[10].

The localization of the assimilatory NR is still questionable[16]. Our observations show that the assimilatory NR in *A. brasilense* was a cytoplasmic enzyme as almost all the NR activity was confined to the soluble supernatant fraction after sonication and centrifugation at 1,20,000 x g.

More studies on assimilatory NR for the improved exploitation of N_2 fixation by this bacterium are necessary since the possibility of using this microbe as a biofertilizer is increasing in tropical soil where nitrate salts are in abundance[17].

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PATENT INFECTION WITH *SCHISTOSOMA INCOGNITUM* IN WHITE MOUSE (*MUS MUSCULUS*)

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IN a study of host susceptibility, Sinha and Srivastava[1] infected white mouse with 300 and 1000 cercariae of *Schistosoma incognitum* and did not find its eggs in the faeces of the mice. They pointed out "In white mouse, though the worms attain maturity, their size was comparatively much smaller and their eggs could not break through the wall of its intestine to be voided with the faeces". The subsequent workers[2–4] supported their view. It was therefore presumed that white mouse is an unsuitable host in which the infection remains in its latent form. For this reason, *patency* could not be taken as criterion for judging immune response in mice—*S. incognitum* model[3–6]. The results of the remark to testify this hypothesis are reported.

Twelve white mice, 4–6 weeks old, of either sex were infected each with 500–600 freshly shed cercariae of *S. incognitum* by tail immersion Method[3]. The cercariae were pooled from twenty naturally infected *Lymnaea luteola* snails which in turn were collected from a big pond in Jabalpur city. On the 30th day of infection and at biweekly intervals thereafter, the faeces of all the mice were collected together in the morning and were divided into two parts. With one part of faeces (1–2 g) routine hatching technique[4] was applied while the other part was processed by acid–ether method[7] for microscopic examination.

After 4 to 6 weeks of infection, six mice were sacrificed to assess the development of the flukes and the period for laying viable eggs. The parasites, so collected, were stained and studied to confirm species of *S. incognitum*[8].

By acid-ether method, the faeces of left over mice (six) were found to contain viable *S. incognitum* eggs on 49th day of infection (figure 1). On the 50th day of infection, the faeces from each mouse was examined

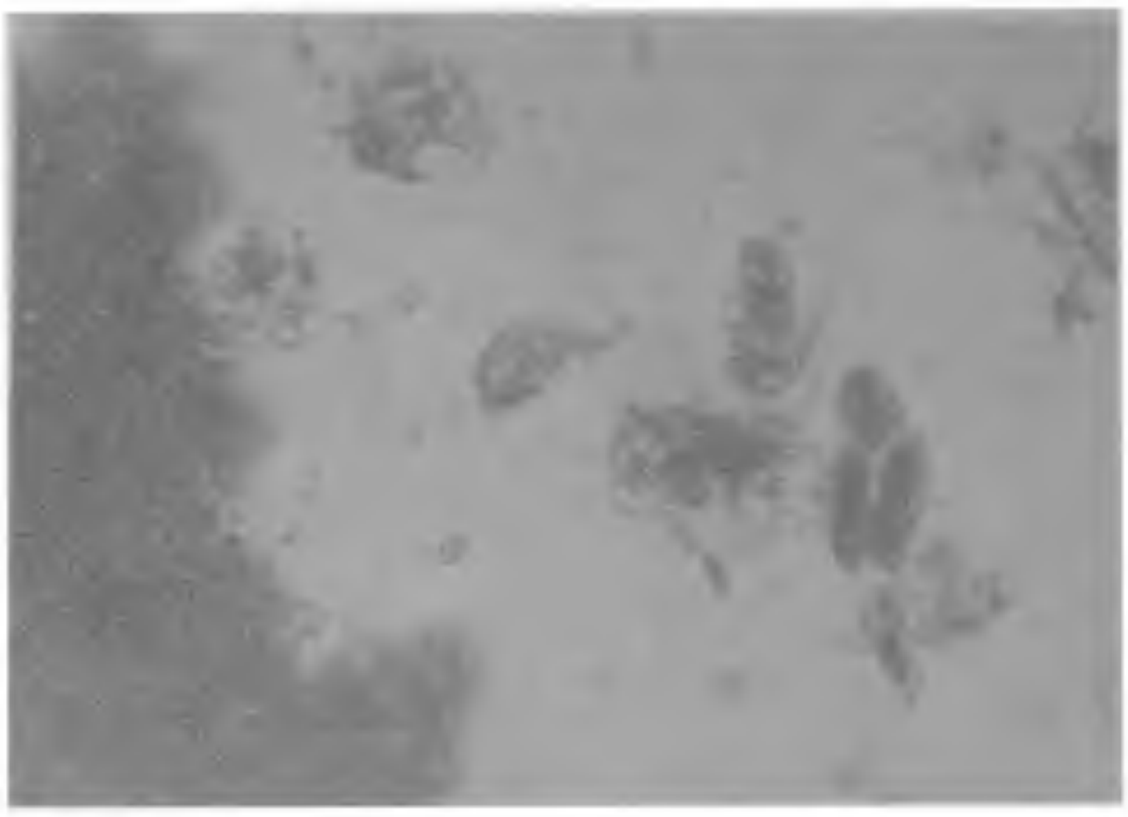

Figure 1. Viable *Schistosoma incognitum* egg in white mice faeces by acid–ether method × 100.

individually and found positive for *S. incognitum* eggs, except one mouse which turned positive on 54th day of infection. The faecal examination by hatching technique and acid–ether method on alternate days was continued till the death of the mice or sacrifice. The number of eggs varied from 300 to 1000 per g of faeces (Mean 400), and diminished in numbers before the death of the animal (table 1.)

The miracidia could not be detected by the routine hatching technique when applied with the faecal samples. According to Lagrange[9], eggs of *S. mansoni* also failed to hatch in the faeces of the albino mouse. Nevertheless, the intestinal washing, taken during postmortem, showed large number of the miracidia, in the present studies.

Two main reasons are responsible for failure of previous workers[1–4] in detecting *S. incognitum* eggs in white mouse faeces. First, they started examining the faeces on 30th day of infection to find the eggs within a week as it happens in pigs. Secondly, hatching technique was relied upon more to detect the infection and no specific method like acid-ether was applied for faecal examination. Naturally, the early reports of absence of eggs in the faeces also deterred the workers to believe otherwise. In the present studies, acid-ether technique was used to a greater extent with the careful examination of all the faecal sediment; the examination was continued for a long period inspite of early negativeness of the faeces. However, existence of a new strain of *S. incognitum* can not be ruled out as the snails were collected from a new pond.

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