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# Animal-Biomedicine Controls Root-Knot-Disease in Lentil-Callus-Culture: Enriched Advanced-Clinical-Toxicology Socio-Economy Science-Technology-Communication by Preventing 21st-Century-COVID-19-Like-Pandemic-Diseases

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## Abstract

The economic pulse seeds, Lens esculentum L. Cv. 'Asha' root callus develop on Murashige and Skoog (MS)-media supplemented with 2 mg/1, 2,4-D (2,4-dichloro phenoxy acetic acid) and inoculated with Meloidogyne incognita larvae (65 ± 2 J2) in the culture tube to study their multiplication rate under sterilized condition in the tissue culture laboratory, and biomedicine-nematode extract (NE) prepared from M. incognita females, when applied at 1.3mg/culture tube to root callus of lentil, reduced nematode infestation of callus and improved callus growth. This animal biomedicine-NE is thought to induce defense-resistance in lentil root callus and may prevent 21st –century pandemics like virus diseases by boosting immunity, resisting toxic effects, and improved biomedicine, advanced in clinical toxicology, green-socio-economy, science technology, and communication application. The biomedicine-NE not only increase agriculture and crop production but also enable the development of a new-generation of nanotechnology-agrochemicals reducing negative-environmental-impacts and maintaining-crop-yields. And both; biomedicine-NE and pulses may itself focus on nanoformulation of agrochemicals and other nanotechnological interdisciplinary future nanotoxicology research and applications in agriculture, and interaction nanomaterials-NE with plants, their environment, and other organisms also.

**Keywords:** Animal-Biomedicine; Controls-Root-Knot-Disease; Lentil-Callus-Culture; Enriched Advanced-Clinical-Toxicology-Socio-Economy-Science-Technology-Communication; Preventing 21st Century-Pandemic

## Introduction

The root-knot nematodes *Meloidogyne incognita* (Kofoid & White) Chitwood infest almost all kinds of vegetable crops, cash crops, and pulses [1-8]. Biomedicines from plant origin being made these days to shift from the conventional use of chemicals to the use of eco-friendly botanicals for the control of root-knot nematodes and easily biodegradable but it has some problems about availability in large quantities from natural sources, isolation of the effective metabolites and it is too much expensive [2-9]. This indiscriminate use of plant resources has already created the problems of biodiversity conservation [10]. Damage caused by root-knot nematodes varies due to several environmental factors and soil types

[11]. To overcome these problems it has been primarily observed under strictly sterilized controlled conditions in tissue culture laboratory that biomedicine from animal origin (Nematode Extract-NE) reduces *M. incognita* infestation in different plants and root callus [5-9] by using their defense response against root-knot infection [12,13]. The present study describes culture, multiplication rate, and control of *M. incognita* on root-callus of lentil grown in well-defined sterile media.

## Materials and Methods

### Root Callus Culture

For the test on root callus, healthy seeds of Lens esculentum L. Cv. 'Asha' were taken into a sterile vial and surface sterilized with 0.1% $HgCl_2$ for 8 minutes. Seeds were then washed thoroughly five times with sterile double distilled water to remove the traces of $HgC_2$ in the laminar flow hood UV-chamber. These seeds were cultured on MS-basal (Murashige & Skoog medium) media (adjusted to pH 5.8 before autoclaving at 120°C for 20 min) in a glass tube (25 x 150 mm) for germination [14] in the tissue culture room, Department of Botany, Visva-Bharati University, Santiniketan, West Bengal, India, where the seeds were incubated at an optimum temperature of 25 ± 2°C and relative humidity of 75 percent. The cultured test tubes were sealed using a cotton plug. After 17 days, the root of the germinated seeds were excised and transferred to callusing MS media (20 ml vol.) supplemented with 2,4-D (2 mg/1). Culture was maintained in glass tubes at 25 ± 2°C with a 16 h photoperiod (General Electric cool white tubes of 50 p Em-2S-2 at test tube level) and relative humidity of 75%. Callus initiation took place within 5 to 7 days from the cut ends as well as the tip of the root. After 15 days, callus growth was vigorous and color was pinkish-white and transferred all the callus into the freshly prepared same callusing media for long culture [15]. Each experiment was repeated five times.

### Preparation of Biomedicine-Nematode Extract (NE)

Large numbers of M. incognita females were collected from pots grown with lentil plants in the garden of the Department of Zoology, Visva-Bharati University, Santiniketan, West Bengal, India. Roots were finely torn into pieces by blender and hand-picked from infected galled-root in cold room (4°C ± 2°C). They were washed repeatedly with sterile distilled water and homogenized in a tissue homogenizer for 5min and then extracted with 90% ethanol in a cold room (4°C ± 2°C) for 5 days. After extraction with ethanol, the entire supernatant was evaporated and the entire homogenate was dried and the residue was kept over anhydrous calcium chloride for dehydration and stored at 4°C. The residue was mixed with sterile distilled water just before application on root callus [5-7].

### Mortality Test

The nematode extract (NE) was dissolved in distilled water at 1.3 mg/0.1 ml. Water suspension of 50 *M. incognita* juveniles was placed in cavity blocks. The water was withdrawn and immediately replaced with test solution from the cavity blocks, except for the control. Juvenile ($J_2$) mortality was determined every 10 min for a period of 80 min. The control was simultaneously observed for juveniles mortality [5-7,16]. The experiment was replicated five times.

### Nematode Sterilization

For the nematode pure culture maintained on lentil in pots; the egg masses of M. incognita were handpicked and transferred to an embryo cup with sterile distilled water and kept for hatching at an ambient temperature of 28 ± 2°C [5-7]. The hatched-out larvae were concentrated by transferring them to sterile tubes and allowing for an hour. The supernatant was decanted and 10 ml of streptomycin sulfate was added to the tubes under the laminar flow hood for minutes. The suspension was equally distributed to the micro-centrifuge tubes and centrifuged for 4 minutes at 3,500 rpm. The supernatant was then decanted and 2 ml of 0.1 percent $HgCl_2$ was added and wait for 10 minutes and again centrifuged at 3,500 rpm for 4 minutes and wait for 10 minutes. After decanting the supernatant 3 ml of sterile distilled water was added and centrifuged at 3,500 rpm for 4 minutes. Likewise, sterile distilled water washes were given five times under the laminar flow hood [5-7,17]. The pellet was then collected in a sterile graduated Pasteur pipette and placed in a sterile dish and to this 5 ml of sterile distilled water was added. With a sterile syringe, 0.1 ml of suspension was syringed out in a sterile BPI dish and the number of the nematodes were counted under stereo zoom, which indicated that 0.1 ml of the suspension contained 65 $J_2$ ± 2 nematodes [5-7,17,18].

### Inoculation

Culture tubes containing lentil root callus were inoculated with nematode suspension in sterile water (0.1 ml) at the rate of 65 ± 2 nematodes ($J_2$) per tube. The cultured test tubes were sealed airtight using a cotton plug and incubated at 25 ± 2°C with 16 h light (General Electric cool white tubes) of 50 p Em"2S"2 at test tube level and 75 percent relative humidity [5-7,17,18]. The culture tubes were divided into batches of ten in each treatment, except inoculated untreated (20 in number); Uninoculated untreated, Inoculated untreated, NE-pretreated and Inoculated, Inoculated and NE-post treated. Inoculated untreated batch contain 20 culture tubes

due to assess the penetration rate in the root callus and ten inoculated culture tubes were taken for those purposes [5-7].

### Treatment

Dehydrated biomedicine-NE mixed with sterile distilled water was introduced into the root-callus and media of culture tubes at the rate of 0.1 ml/treated tube (containing 1.3 mg of dehydrated NE) 3 days before and 3 days after inoculation with nematodes ($J_2$) for Pre-and Post-inoculation treatments respectively. Control tubes were treated with an equal amount (0.1 ml) of sterile distilled water. All inoculation and treatments were done in an aseptic condition. Ten number of the root calli of inoculated untreated culture tubes batch were taken out 20 days after inoculation for the asses of infection rate [5-7]. All the root calli were taken out 47-days after inoculation and the following parameters were observed: biomass of root callus, number of juveniles in callus and media, number of mature females in callus, ratio of initial and final population, and estimation of protein in root callus [19,20]. All the data were analyzed by ANOVA (Analysis of Variance). The experiment was repeated five times with similar results. Data from the third experiment are reported here.

## Results

Callus initiation took place within 5-7 days from the cut ends as well as the tip of the root (Table 1). After 15 days, callus growth was vigorous and the color was pinkish white. The inoculated untreated lentil root callus were taken out and tested out calli on the 21st day of inoculation. The third stage of juveniles ($J_3$) was maximum (Table 1). There was 49.86 percent larval penetration in callus and 0 percent in the media. All the inoculated pre- and post-treated root callus turned light pink color to light brown and loose-friable in texture after 27 to 29 days of inoculation and those turned dark brown after 44 to 47 days. In lentil root callus, the uninoculated and untreated root calli as well as inoculated treated ones had significantly greater biomass than inoculated untreated calli (Table 1). The number of juveniles and mature females was significantly reduced in treated calli compared to the inoculated untreated calli. Nematodes multiplication rate increase in the inoculated untreated callus (106 percent) and inoculated post-treated (10.76 percent) but decrease in inoculated pre-treated callus (7.69 percent). The ratio of initial and final population was highest in pre-treated calli (1.08) but lowest in inoculated untreated calli (0.49) and next was post-treated calli (0.90) Juveniles in media were higher (57.14%) in number than those in calli of pre-treated tubes. In the case of post-treated tubes, calli contained more juveniles (9.37%) than the media. In the case of inoculated but untreated tubes, juveniles in media were nil. Pretreatment effects were better than the post-treatment effects in terms of nematode population in root callus and media (Table 1). Biomedicine-Nematode extract (NE) had not produced any direct toxic effect on nematode because no mortality occurs after 80 minutes exposure period at 1.3 mg/0.1 ml concentration. The Biomedicine-NE treated (1.3 mg/tube) callus did not show any toxic effect also.

| Treatment | Biomass of Callus'x' (g) | Infection Rate/ callus'x' (%) (at day-21) | Number of Juveniles 'x' in | | Number of Females'x' In Callus'xx' (2g) | Ratio 'x' (Initial/final population) | Callus protein'x' content percent |
|---|---|---|---|---|---|---|---|
| | | | Root Callus'xx' (2g) | Medium'xx' (20 ml) | | | |
| Uninoculated Untreated | 2.35a ±0.05 | 49.86% | - | - | - | - | 3.99 c ±0.01 |
| Inoculated Untreated | 1.55c ±0.03 | - | 102 a ±2.85 | - | 32 a ± 1.02 | 12.49.00 AM ± 0.01 | 5.78 a ±0.08 |
| Pretreated(NE) Inoculated | 2.40a ±0.02 | - | 21 c ±0.09 | 33 a ± 1.25 | 6c ± 0.01 | 1.08c ± 0.02 | 4.01 c ±0.02 |
| Inoculated Post treated(NE) | 1.98b ±0.03 | - | 32 b ± 1.02 | 29 b ± 1.25 | 11b ± 0.35 | 0.90b ± 0.02 | 4.56 b ±0.02 |

**Table1.** Effects of the biomedicine-nematode-extract (NE) on the biomass of lentil root callus (*Lens esculentum* L. Cv. 'Asha') inoculated with *M. incognita* (65 J2 ±2 / callus), number of juveniles in callus and MS media, number of females in callus, callus protein content percent, ratio of initial and final population and infection rate of juveniles at Day-21.
**'x'** - Mean of ten replicates with S.E.
**'xx'**- Means carrying some letters in a column are not significantly different (P-0.05).
**' –'** - Mean absent or not applicable.

## Discussion

Root callus of lentil Cv. 'Asha' initiation took place within 5 to 7 days and after 15 days callus growth were vigorous due to nutrient in Murashige & Skoog medium with growth regulator (2 mg/1, 2,4-D) for callus induction [5-7,13,17]. It is reported that in spite of permitting feeding of *M. incognita* the tomato root callus did not support its development or multiplication as on 45.33 percent larvae penetrated the callus and 27.94 percent reached the adult female stage [17]. For these reason in present experiment *M. incognita* juveniles penetration rate was 49.86 percent after 20 days of inoculation in the lentil root callus. I did not get any adult females in the callus at day-21, because third stage of juveniles (J3) were present in large number though large number of mature females were observed at day-48 and the multiplication rate was 50.76 percent. All the inoculated, pre- and post-treated callus turned light pink to light brown after 29 days of inoculation due to indication of normal feeding and different development of the nematodes and those were turned dark brown (44 to 47 days) due to excessive feeding of nematodes as well as suitability of the callus for the development of mature *M. incognita* females [5-7,13,17].

The present experiment clearly showed that nematode extract (NE) was treated as effective biomedicine from animal origin and it had no toxic direct effect on nematodes and root callus. It was not only control *M. incognita* population in root callus but also induced some resistance in lentil root callus to *M. incognita* infection. The pre-treatment was more effective than the post-inoculation treatment [5-7].

Result of the root calli tissue culture further indicated that biomedicine-NE-treatment could promote growth of root callus and the resistance induced by NE was systemic. Since large number of *M. incognita* larvae were present in MS media in case of both treated calli and were totally absent in media of untreated cultures. It can be assumed that NE could induce synthesis of some antagonistic substance in the treated calli [5-7,13]. Lectins accumulated in galled regions of root of *Hibiscus esculentus* infected with *M. incognita* [21]. Systemic acquired resistance can be induced in different crop plants by localized virus infection, non-pathogenic and pathogenic microorganisms or their culture filtrates or by salicylic acid. This form of resistance protects plants from a broad spectrum of pathogens and work systemically in many cases [5-7,13,22-30].

*M. incognita* is known to share common antigens with its host plants [31]. It appeared that during natural infection with the nematode, host plants showed minimal defense responses to the nematode because of this antigenic similarity. Biomedicine-Nematode extract (NE) containing various antigens may induce defence responses involving a number of pathogenesis related protein in which the nematodes fails to tolerate [5-7]. Datta (1999) observed that in lady's finger plants treated with Biomedicine-NE showed the highest number of root protein (23 and 24) but inoculated untreated root was 18 number and uninoculated untreated root was 11 number [7]. Those showed that NE served as a stimulus for the expression of many proteins particularly the defence-related protein which latter provide resistance to nematode infection. However, in the test plant were treated with the NE after inoculation with live nematode did not show much increase in number of protein (16) in root [7]. Those showed that nematode infestation somehow serve as a repressor for the expression of defense gene in plant.

In plant-nematode interaction, newly synthesized proteins including PR-protein have been found in potato plants infected with the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* [32-34]. Nandi, et al., (2002, 2003) reported that salicylic acid enhances resistance in cowpea and okra plants against *M. incognita* by inducing expression and accumulation of pathogenesis related-I protein (14 KD, PR-I) in sprayed plant root and leaves. They also reported that SA spray enhances PAL higher activity in infected roots and it has been suggested in plants nematode-extract-induced systemic resistance triggers a signal transduction pathway that is different from other common pathogens or bacterial or chemical induced pathways [13,29-35].

Though root-callus culture of root-knot nematodes in well defines media is in vogue already and the methods, media preparation are elaborate, complex, and costly and time consuming [5-7,13]. But in the present experiment it was very effective because once again it is confirmed our earlier observations in root callus nematode culture in well-defined sterile media where there were no interference of environmental factors and contamination. By the application of the animal biomedicines-NE, it is not only controlled root-knot nematodes and growth of callus or plants by inducing their defense response in field application, but also conserved biodiversity and makes pollution free environment.

## Future Research

In future both; the lentil itself and synthetic production of biomedicine-NE will be the potential cost-effective personalized-biomedicine OR social vaccine OR vaccine against COVID-19 like pandemic virus diseases for its benefit with nutritional recommendations for CoVID-19 quarantine by increasing immunity, and helping policy or strategies, removing the harmful effects of nanoparticles on animals and plants, and clinical research in all areas of advanced toxicology, immunotoxicity, neurotoxicity and drug toxicity etc., and it is effectively applied by the NGO or students [9,36-44].

## Conclusion

The animal biomedicine-nematode-extract reduced nematode infestation of economic-pulse root-callus and improved callus growth by inducing defense-resistance in lentil root callus, and may prevent 21st –century pandemics like virus diseases by boosting immunity, resisting toxic effects, and improved biomedicine, advanced in clinical toxicology, green-socio-economy, science technology, and communication application. The biomedicine-NE not only increase agriculture and crop production but also enable the development of a new-generation of nanotechnology agrochemicals reducing negative environmental impacts and maintaining-crop-yields. And both; biomedicine-NE and pulses may itself focus on nanoformulation of agrochemicals and other nanotechnological interdisciplinary future nanotoxicology research and applications in agriculture, and interaction nanomaterials-NE with plants, their environment, and other organisms also.

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