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Author's Affiliation:
1. Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar - Pakistan
2. Institute of Integrative Biosciences IIB, CECOS University Peshawar - Pakistan
3. University Institute of Medical Laboratory Technology, The University of Lahore-Pakistan

Corresponding Author:
Mian Afaq Ahmad
Email: mianafaq@aup.edu.pk

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Green Nano-synthesis: *Salix alba Bark*-Derived Zinc Oxide Nanoparticle and their nematicidal Efficacy against root knot nematode *Meloidogyne incognita*

Sidra Ahmad[1], Mian Afaq Ahmad[1]*, Fazal Umar[1], Iqbal Munir[1], Neelum Iqbal[1], Nayab Ahmad[1], Gulzar Ahmad[1], Muhammad Ijaz[2], Zia Ur Rahman[3], Syed Jehangir Shah[1], Hamza Iqbal[1], Ateeq Ur Rahman[1]

Abstract

Background: Plant parasitic nematodes (PPNs) are considered major agriculture pests, causing significant damage to crops by directly targeting the plant root system to prevent water and nutrient uptake. One of its major species, *Meloidogyne incognita,* is considered a serious threat to agriculture crop production worldwide. The current study was intended to evaluate the potential of Nanoparticles synthesized from *Salix alba* bark extract as nematicidal agent.

Methods: Phytochemical analyses of *Salix alba* bark extract were conducted, and nanoparticles of the same extract were synthesized and characterized using UV-vis spectroscopy, XRD (X-Ray Diffraction), SEM (Scanning Electron Microscopy) and EDX (Energy Dispersive X-ray Spectroscopy) techniques.

Results: The qualitative phytochemical analysis of *Salix alba* bark extract revealed the presence of phenolics, flavonoids, reducing sugars, and saponins. When applied on juveniles of the plant parasitic nematode *Meloidogyne incognita*, the nanoparticles demonstrated a dose and time-dependent impact. After 24 hours, the highest concentration (1000 µg/ml) of nanoparticles exhibited the greatest mortality, reaching 82.2%, while the lowest concentration resulted in a mortality rate of 34.5%. Similarly, at the 48-hour mark, the highest mortality (92.2%) was observed with 1000 µg/ml of nanoparticles, whereas the lowest concentration yielded a mortality rate of 54.5%. Extending the observation period to 72 hours, the mortality rate peaked at 98.33% with the highest nanoparticle concentration (1000 µg/ml), and the lowest mortality rate was recorded at the lowest dose, amounting to 72.5%. These results underscore the dose and time-dependent efficacy of *Salix alba* bark-derived nanoparticles against *Meloidogyne incognita.*

Conclusion: Our findings suggest that zinc oxide nanoparticles synthesized from *Salix alba* bark can be an effective agent against plant parasitic nematode *Meloidogyne incognita*. Further experiments are recommended to study the impact of these nanoparticles on other biotic stresses.

Introduction

Meloidogyne incognita, commonly known as the root knot nematode, holds considerable economic importance due to its profound effects on crucial crops [1-5]. Representing a staggering 90% of the agricultural damage caused by various nematode species, Meloidogyne incognita exerts a substantial influence on both crop yield and quality [6,7]. Although chemical nematocides, referred to as agrochemicals, are prevalent for safeguarding plants against nematodes, their utilization raises concerns regarding potential adverse impacts on human health [6,8]. Consequently, biological nematocides emerge as a promising alternative for the effective management of plant parasitic nematodes [9,10]. Nanoparticles represent a significant and diverse field with myriad applications across various scientific disciplines [8,11]. Their elevated surface area to volume ratio imparts superior thermal conductivity, antibacterial efficacy, catalytic activity, and chemical stability compared to larger counterparts [11,12]. The adoption of green nanoparticle synthesis, characterized by its environmental sustainability and cost-effectiveness, eliminates the need for hazardous reductants and stabilizing agents in the synthesis process [13,14]. Zinc oxide nanoparticles, among metal oxide counterparts, exhibit versatile applications, encompassing disease diagnostics, drug delivery, and demonstrating antioxidant and antibacterial capabilities [15]. *Salix alba*, colloquially known as white willow and belonging to the Salicaceae family, boasts a medicinal history spanning 6000 years [16,17]. Salicin, identified as a prodrug present in both the leaves and bark of *Salix alba*, commonly known as white willow, is prominently featured in the bark extract. This compound holds considerable pharmacological potential, thereby attracting special attention for further investigation. *Salix alba* is notably rich in polyphenols and flavonoids, compounds recognized for their therapeutic attributes, particularly for their anti-inflammatory and antioxidant effects [18,19]. The medicinal applications of willow bark span a broad spectrum, encompassing its effectiveness in treating inflammation, pain, fever, back pain, knee pain, and arthritis [19]. The diverse therapeutic benefits can be attributed to the high concentration of polyphenols and flavonoids within *Salix alba* [20,21].

Gold nanoparticles derived from *Salix alba* have demonstrated significant antibacterial and antifungal activities against a diverse range of bacterial and fungal species [22,23]. Likewise, silver nanoparticles synthesized through an environmentally friendly approach utilizing *Salix alba* have exhibited notable antibacterial efficacy against various bacterial pathogen[13,22]. These findings underscore the potential of *Salix alba*-derived nanoparticles for antimicrobial applications and warrant further exploration of their mechanisms and potential applications in biomedicine, Agriculture and related fields [24,25]. The current study was intended to evaluate the potential of Nanoparticles synthesized from *Salix alba* bark extract as nematicidal agent.

Methods

Plant Collection and Plant Extract Preparation

Salix alba specimens were systematically gathered, meticulously cleansed, and subjected to shade-drying before undergoing fine pulverization. Subsequently, 10 grams of the resulting plant material were solubilized in 100 ml of deionized water. This solution underwent a rigorous one-hour treatment in an 80°C water bath, followed by a thorough triple-filtration procedure utilizing Whatman filter paper to obtain an aqueous plant extract. The pH of the resulting plant extract was determined using a calibrated pH meter [26].

Phytochemical Evaluation

A comprehensive phytochemical investigation was conducted on the *Salix alba* bark extract, employing a series of experiments aimed at detecting various phytoconstituents. The concentration of flavonoids Test; It was ascertained by adding 1 ml of the plant extract to the apparatus for the resolve of flavonoid concentration we take ten milliliters of distilled water, followed by the sequential addition of five milliliters of ammonia solution and concentrated H2SO4, with each reagent added separately[27]. For the detection of phenolic chemicals, 1 milliliter of the plant extract was combined with five milliliters of Folin-Ciocalteu reagent, and subsequently, four milliliters of sodium carbonate were incorporated into the mixture [28]. To assess reducing sugar, one milliliter of ethanol was introduced to 5 ml of the plant extract in an aqueous medium. Simultaneously, a separate test tube containing 1 ml each of Fehling solution A and B was heated to boiling. The resulting plant extract was then examined for observable modifications, including color changes and precipitate formation [27]. In the case of saponins investigations, one milliliter of the plant extract was amalgamated with 5 milliliters of distilled water, subjected to vigorous shaking, and exposed to high temperature. The presence of bubbles upon heating was indicative of the presence of saponins[29].

Zinc Oxide Nanoparticle Synthesis

The production of zinc oxide nanoparticles employed a tailored methodology derived from the approach detailed by[26]. Specifically, 6 grams of zinc acetate dehydrate were introduced into 100 ml of plant extract. The resulting solution underwent a two-hour heating process at 60°C with continuous agitation. Subsequent

to heating, the pH of the solution transitioned from 5.3 to 5.8. Following a 20-minute centrifugation at 10,000 rpm and 40°C, a precipitate was obtained. This precipitate underwent dual washing with distilled water at 10,000 rpm for 15 minutes to eradicate impurities. The resultant supernatant was discarded, leaving a particle that underwent drying in an oven. Utilizing a mortar and pestle, the desiccated precipitate underwent meticulous crushing, yielding a refined powder. This powder underwent annealing in a 500°C furnace for 2 hours to improve stability and purity, giving nano powder for further analysis.

Nanoparticle Characterization

The comprehensive examination of the synthesized zinc oxide nanoparticles involved rigorous scrutiny employing various scientific methodologies. UV-Vis Spectrophotometric Analysis entailed the precise 1:2 dissolution of the biosynthesized nanoparticle solution in deionized water, with deionized water serving as the baseline. Spectrum scans across the 350-450 nm wavelength range were conducted utilizing the UV-1700 Pharma Spec apparatus [30]. (XRD) analysis utilized the dried nano powder derived from zinc oxide nanoparticles. The crystalline structure and average size of the nanoparticles were determined employing the JEOL Japan Model JDX 3532. (SEM) analysis; utilizing the JSM-IT100 JEOL SEM model, provided insights into the morphology and shape of the biosynthesized zinc oxide nanoparticles. (FTIR) Analysis: For the functional group identifications (FTIR) analysis, enabling the acquisition of an infrared absorption and emission spectrum. This analytical approach played a crucial role in identifying specific functional groups and discerning the involvement of various phytochemical elements in the stabilization and reduction processes of the nanoparticles.

(EDX) Analysis

The elemental composition of the synthesized nanoparticles underwent assessment through Energy Dispersive X-Ray Spectroscopy (EDX) analysis, utilizing the capabilities of the UK Model INCA 200. This analytical technique provided valuable insights into the elemental constituents of the produced nanoparticles[31,32].

Nematicidal Behavior

Root Sample Collection

To conduct the mortality assay, root samples hosting root knot nematodes (RKNs) were acquired from three-month-old tomato plants. These samples, recognized based on morphological characteristics of adult females as documented by [33], were securely stored in plastic bags.

Egg Harvesting

The infested root samples underwent a two-day immersion in water to facilitate the softening of galls. Following this period, the roots were precisely sectioned into small fragments using scissors. These root segments were then finely ground using an electric grinder in a 1.5% sodium hypochlorite (NaOCl) solution for 30 seconds, followed by a thorough rinse with water. To extract eggs, the ground solution underwent sequential sieving starting from 550 µm, then 300 µm, 250 µm, and up to 6 different concentrations, down to 25 µm. Second-stage juveniles (J2s) were separated by filtering the collected eggs through paper. Confirmation of these juveniles was achieved through meticulous examination under a stereo microscope, and subsequently, they were cultured at 28°C for future experimentation [6].

Dose-Response of *Meloidogyne incognita* to ZnO Nanoparticles

Second-stage juveniles (J2) of *Meloidogyne incognita* were exposed to varying concentrations of zinc oxide nanoparticles (ZnO NPs). About 200 nematodes were introduced into 1 ml of distilled water containing 100 µg/ml, 500 µg/ml, and 1000 µg/ml of ZnO NPs, along with a plant extract solution; distilled water served as the control. Nematicidal activity was assessed using a stereo microscope at 24, 48, and 72 hours for each concentration, with counting facilitated by a counting plate. The experiment was replicated three times, and juvenile mortality was determined using a predefined method [34].

Statistical Analysis:

Involved Origin Pro 8.5, Microsoft Excel, and SPSS, with ANOVA followed by Dunnett's multiple comparison test, considering a significance level of $p \leqslant 0.05$.

$$\text{Mortality} = \frac{\text{number of dead juveniles}}{\text{total no of juveniles}} \times 100$$

Results

Phytochemical Analysis

Phytochemical analysis was done to pinpoint the present Phytochemical in our plant extract they are shown in Table 1 shows the confirmation with concentrations these compounds of different levels of flavonoids, phenolics, reducing sugars and saponins in plant extract of *Salix alba*. In flavonoids test, solution color was changed to yellow while in phenolics solution color changed to blue. Reducing sugars were detected by precipitate formation and saponins by bubbles formation.

Phytochemical test	Intensity
Flavonoids	++
Reducing sugars	+++
Phenolics	+++
Saponins	++

Table 1: The highest Levels of bioactive compounds in *S. alba* extract is phenolics and Reducing Sugar followed by Flavonoids and Saponins.

Initial Color Change Upon Synthesis of Nanoparticles

When zinc acetate dehydrate was added to the aqueous extract of *Salix alba,* upon constant shaking. Its color was changed to orange from brown (Fig. 1A). This change in color indicated preliminary synthesis of zinc oxide nanoparticles which was also reported by [35]. (Fig, 1B) Root samples of tomato plants infected with root knot nematodes showing galls.

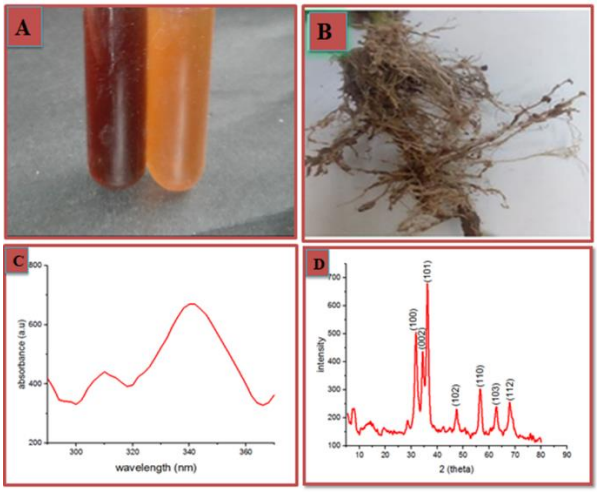

Figure 1: (A) the Color transformation of *Salix alba* extract is the indications of the creation of nanoparticles from dark-brownish to light-orange **(B)** Root samples shows the formations of gall in the infected plants of tomato with PPN **(C)** It shows UV-the peak 342 nm of Zinc Oxide Nanoparticles of *Salix alba* **(D)** The X-ray diffraction different angle is the validation of crystalline structure of *Salix alba* nanoparticle.

Characterization of ZnO nanoparticles

The zinc oxide nanoparticles underwent a comprehensive characterization employing UV-vis, XRD, FTIR, SEM, and EDX techniques. UV-vis analysis, utilizing spectrophotometry, investigated the light absorbance of *Salix alba* nanoparticles, revealing a prominent peak at 342 nm (Fig. 1C). This absorbance, falling within the typical range for zinc oxide nanoparticles (320 nm to 380 nm), served as confirmation of their successful synthesis. *Salix alba* X-Ray Diffraction Analysis: exhibited well-defined peaks at 31.85°, 34.46°, 36.25°, 47.49°, 56.59°, 62.76°, and 68.12° (Fig. 1D), corresponding to crystalline planes (100), (002), (101), (102), (110), (103), and (112). This pattern unequivocally verified the crystalline nature of the green synthesise ZnO nanoparticles, aligning with data from JCPDS file no. 361451. The calculated average nano-crystallite size, determined using Debye's Sherrer formula, was 7.14 nm.

FTIR Examination

The identification of functional groups involved in the reduction process was conducted through FTIR spectroscopic evaluation of zinc oxide nanoparticles and *Salix alba* plant extracts. In (Fig. 2A) distinctive patterns at 991 cm^-1, 1380 cm^-1, 1576 cm^-1, and 3300 cm^-1 were observed, potentially indicating the presence of alkene, phenol, amine groups, and hydroxyl groups, respectively. A comparative analysis was performed with the plant extract (Fig. 2B). SEM results revealed internal compositions that were employed for morphological assessment of *Salix alba* nanoparticles at various resolutions. The SEM data (Fig. 2C&D) revealed that *Salix alba* nanoparticles exhibited irregular shapes and agglomeration.

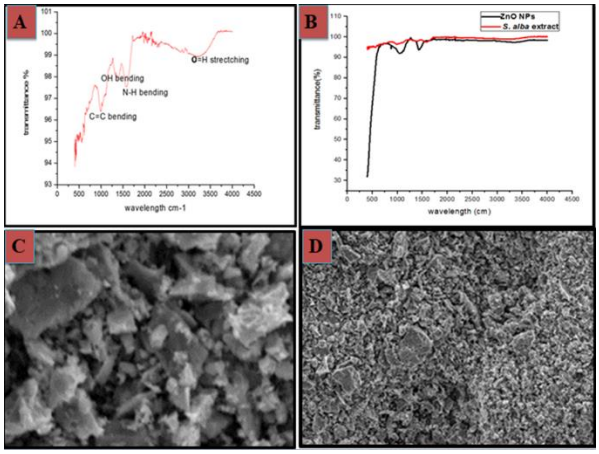

Figure: 2 (A) FTIR examination of *Salix alba* bark extract showing the functional groups **(B)** FTIR investigation showing evaluation of *Salix alba* extract with ZnO NP **(C)** SEM photographs of ZnO nanoparticles of *Salix alba* **(D)** Indicating the nanoparticles irregular shape.

EDX Analysis

The EDX spectrum of *Salix alba* nanoparticles showed highest peaks of zinc and oxygen (Fig. 3A&B). The percentage of zinc element was 80.39 % and oxygen was 16.08 %. These results confirmed the synthesis of zinc oxide nanoparticles. Nematicidal Activity; *M. incognita* juveniles obtained from tomato roots were treated to various dosages of *Salix alba* ZnO NP. These nanoparticles exhibited efficient nematicidal action against *M. incognita* 2nd stage juveniles (Fig. 3D&C).

Meloidogyne incognita Mortality in Response to *Salix alba* Extract and Nanoparticles

The bark extract of *Salix alba* exhibited significant activity against second-stage juveniles of M. incognita, resulting in mortality rates of 25.83%, 42.2%, and 50.2% after 24, 48, and 72 hours, respectively (Fig. 4).

Number of Dead and Alive Nematodes						
Treatments	**After 24 hours**		**After 48 hours**		**After 72 hours**	
	Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**
Control	180.3±0.94^{a}	21.7 ±2.49^{e}	171.0±4.1^{a}	31.7±3.26^{e}	164.3±4.32^{a}	29.0±2.49^{d}
Plant extract	124.3±8.99^{b}	51.7±3.39^{d}	115.0±1.63^{b}	84.3±3.29^{d}	103.6±4.98^{b}	100.3±6.18^{c}
100 µg/ml	129.3±5.79^{b}	69.0±7.11^{c}	88.7±1.69^{c}	109.0±4.32^{c}	27.6±7.71^{c}	145.0±7.11^{b}
500 µg/ml	89.7±5.25^{c}	115.7±6.18^{b}	61.7±6.79^{d}	153.0±10.03^{b}	25.0±5.88^{c}	185.0±3.26^{a}
1000 µg/ml	41.7±5.25^{d}	164.3±5.43^{a}	13.7±0.94^{e}	184.3±4.10^{a}	3.33±1.24^{d}	196.7±1.24^{a}

Table 2: Effects of *Salix alba* bark extract and zinc oxide nanoparticles on J2s of root knot nematode *Meloidogyne incognita*. Each value represents the mean ± standard deviation of three replicates. Data were subjected to ANOVA followed by Tukey HSD comparison test. All treatments were significantly different from control group ($p<0.05$).

Additionally, *Salix alba* nanoparticles were tested at concentrations of 100 µg/ml, 500 µg/ml, and 1000 µg/ml to assess juvenile mortality over different time intervals (24, 48, and 72 hours). The nanoparticle concentrations showed increasing mortality rates: after 24 hours (34.5%, 57.8%, and 82.2%), 48 hours (54.5%, 76.5%, and 92.2%), and 72 hours (72.5%, 92.5%, and 98.3%) at 100 µg/ml, 500 µg/ml, and 1000 µg/ml, respectively (Fig. 4).

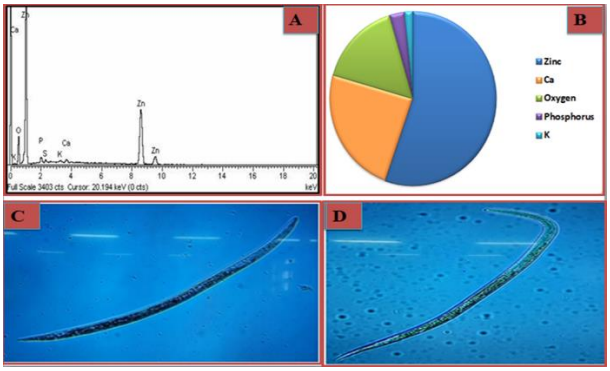

Figure 3: (A) EDX bands of *Salix alba* nanoparticles show elemental Analysis. **(B)** pie chart also showing presence of zinc, Ca and oxygen in high Amount **(C)** in this figure the straight nature and no movement is the indication of dead *M. incognita* **(D)** in the other side the proper movement of Alive root knot nematode *M. incognita.*

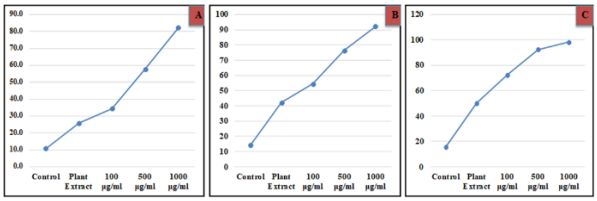

Figure 4: Mortality Rate in percentage after several intervals (%) after **(A)** 24 hours **(B)** 48 hours and **(C)** 72 hours.

Discussion

In this study, *Salix alba* bark extract was used to create zinc oxide nanoparticles using a green method. In green synthesis phytochemical constituents of plants play a very crucial role in the synthesis process. *S. alba* extract contains phenolics, flavonoids and saponins. Because these phytochemicals have an OH group, which FTIR analysis also confirms, they function as reducing and stabilizing agents during the nanoparticle synthesis process [36,37]. While flavonoids and saponins were present in moderate amounts, *Salix alba* displayed high levels of phenolics and reducing sugars (Table 1).

Initially, synthesis process was confirmed by color change. A change in color upon synthesis of nanoparticles was also observed by [38] which supports our results. The formation of pure zinc oxide nanoparticles is indicated by the absorption peak these nanoparticles displayed, which was observed in the range of 360 to 380 nm [39]. This absorption peak is most likely the result of zinc oxide band gap absorption brought on by electrons moving from the valence band to the conduction band [40]. The XRD pattern demonstrated the crystalline nature of the *Salix alba* biosynthesized zinc oxide nanoparticles when compared to the reference JCPD file. Because there were no impurity peaks in the XRD pattern, the produced zinc oxide nanoparticles were shown to be pure. Our results' interpretation is consistent with that of Pillai *et al.*, who determined the crystalline size using the FWHM value and similar XRD peaks [41].

Our phytochemical analysis of *Salix alba* is supported by the presence of functional groups, as shown by FTIR analysis (Table 1). When the FTIR spectra of *Salix alba* zinc oxide nanoparticles and plant extract were compared (Fig. 2B), it was shown that a band at 3300 cm-1 in the zinc oxide nanoparticle spectrum had vanished. The hydroxyl group is represented by the O-H stretching in this band at 3300 cm-1. The band's disappearance indicated that phytochemicals were involved in the *Salix alba* nanoparticles' synthesis. This OH group, which is primarily found in flavonoids and phenolics, serves as a capping, reducing, and stabilizing agent when nanoparticles are being synthesized [26] .

SEM analysis revealed the irregular morphology of zinc oxide nanoparticles, accompanied by observable particle agglomeration during the synthesis process. This agglomeration phenomenon is attributed to the inherent high surface energy of zinc oxide nanoparticles, a common characteristic in the realm of green nanoparticle synthesis[26]. Additionally, the EDX spectra exhibited an additional signal associated with calcium, likely stemming from the use of deionized water in the nanoparticle synthesis process—a finding consistent with similar observations reported by others [42]. The presence of phytochemicals in the plant extract, acting as stabilizing and capping agents, offers a plausible explanation for the emergence of these supplementary peaks [26].

The investigators observed that nanoparticles derived from *Salix alba* exerted a dose- and time-dependent inhibitory effect on the development of Meloidogyne incognita juveniles. (Fig. 4). [4] also reported nematicidal properties of nanoparticles in dose dependent manner. When concentration and time period was increased, the toxicity of synthesized zinc oxide nanoparticles was also increased. Al Banna *et al.*, [43] concluded that sulfur nanoparticles are toxic against *Meloidogyne javanica*. Earlier it was evaluated nematicidal activity of *Penicillium chrysogenum* (Snef1216) against *Meloidogyne incognita*. They also concluded that mortality of juveniles was increased by increasing concentration and time period. So biological pesticides can be very useful alternative for harmful chemical pesticides [44].

Our results suggest that biosynthesized zinc oxide nanoparticles showed great potential to be used as biological nematicide for the control of plant parasitic nematodes, ultimately preventing huge crop losses. This approach seems to be ecofriendly and efficient against the tested *M. incognita* juveniles. Further, experiments can be performed to study their impact on other pests and insects.

Conflict of Interest

To avoid any conflict of interest we have removed almost all of the plagiarized work but as for its to be mention that this thesis has submitted only for degree requirements purposes to The University of Agriculture Peshawar Pakistan that's our own work which was not published before not a part of it to any other journals except to only AUP QEC departments that's why the plagiarism report shows the similarity indexed 45 % from which 39 % is with our own work which is only in the database of Turnitin not Published.

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Author Contributions

This work has been done under the kind supervision of MA and IM. SA and FU performed research, NI and NA analyzed the data, GA and IA helped in characterization, SJH and HI collected plant and nematode samples, ZUR and AR helped in proofreading. SA, IA & GA wrote the first draft and edited the manuscript. All authors read and approved the final manuscript.

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