

Salicylic acid mediated defense response against root-knot nematode in tuberose

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Abstract

Root-knot nematode (*Meloidogyne incognita*) poses a considerable threat to tuberose cultivation. Biological compounds, such as salicylic acid (SA), act as systemic acquired resistance (SAR) elicitors and may contribute to the resistance of host plants against nematodes. The study investigated the effects of SA at varying concentrations on inducing resistance in tuberose against *M. incognita* via tuber soaking, foliar spray, or a combined method. Plant growth parameters indicated that pretreatment of the SA exhibited no significant negative effect on the shoot length, shoot weight, number of leaves, tuber weight and root weight. The root image analysis revealed a positive effect of 10 mM SA on root length, number of root tips, forks, and crossings compared to the inoculated control plants. Leaf spray with SA is the most effective treatment, as it markedly reduces both root galling (54.8-71.6%) and infestation (66.8-72.6%). Using the simple, quick, and cost-effective SA spray approach, a 64 to 76% reduction in the multiplication factor (MF) of nematode infestation was observed. This is the first evidence of the use of SA in tuberose to induce resistance against *M. incognita*.

Keywords: Salicylic acid, tuberose, *Polianthes tuberosa*, systemic acquired resistance, root knot nematode, *Meloidogyne incognita*

Introduction

Plants sessile nature exposes them to an array of environmental stresses. *Polianthes tuberosa* (tuberose) is an economically important crop cultivated all over the world for its alluring florets with popular properties. It is used for making superior quality scents, cosmetics, garlands and bouquets. The plant is mainly propagated by vegetative means, which paves way for entry of numerous pathogens. Among these, plant parasitic worms, commonly called as root knot nematodes (RKN) (*Meloidogyne incognita*) contribute profoundly for the drastic reduction in the plant productivity (Khan and Parvatha Reddy, 1992) and delays spike emergence (65.07 and 68.33 days). The presence of root knot nematodes facilitates the intrusion of other pathogens as manifested in the reduced growth and tuberose yield. Eventually, RKN infestation in tuberose worsens, as it goes unrecognized until the plant is completely destroyed.

Conventional approaches employ nematicides to control the nematodes. However, the high cost of these compounds and their associated detrimental environmental effects lead to their reduced availability in recent years. Hence, there is an immediate urge for the alternative and reliable sustainable management strategy for combating nematode problem. For instance, methyl bromide is an important fumigant for the control of nematodes, weeds, fungi and other broad range of organisms that has been phased out of several developed countries in view of its significant contribution in Earth's ozone layer depletion (Carpenter *et al.*, 2001). The U.S. law prohibits the sale of neurotoxic nematicides products *i.e.* fenamiphos and carbamate since 2007 (Li *et al.*, 2011). Another extensively used strategy to tackle nematodes is crop rotation. While traditionally used to counter many biotic stresses its effectiveness is limited in many cases. RKNs are capable of infecting a wide range of plants, targeting both monocot and dicot plants thereby obstructing the control strategy via crop rotation.

Moreover, *M. incognita* is able to survive deep within the tuberose bulbs for a long period of time (Chawla *et al.*, 2006) again limiting the crop rotation strategy. Further, the absence of resistant crop varieties on the market bench triggers the need to have advanced eco-friendly and financially sound strategies.

Salicylic acid (SA) treatment is a sustainable method that works by inducing systemic resistance in plants to protect against the pests. It is a plant hormone that acts as a growth regulator affecting different metabolic and physiological processes like transpiration, photosynthesis, transportation and ion uptake (Ibrahim *et al.*, 2019). Exogenous use of SA increases the yield of vegetable crops by minimizing the growth reduction induced by stress (Khan *et al.*, 2015). The potential mechanisms by which SA may exert growth promoting effects are ascribed to changes in hormonal status or by improving the transpiration, photosynthesis and stomatal conductance along with osmoregulation and enzyme activities of antioxidants (Faried *et al.*, 2017; El-Hady *et al.*, 2021). Although SA was once considered to be a relatively trivial product, its role in plant defense response has only recently been recognized. The interest in SA as a disease resistant agent spiked only following the findings that SA treated tobacco plants showed resistance to Tobacco mosaic virus infection (Klessig *et al.*, 2018). SA originates in the plant leaves and the inflorescences upon pathogen attack and it is biosynthesized from chorismate via two pathways, isochorismate synthase and phenylalanine ammonia lyase dependent pathways (Dempsey and Klessig, 2017). Apart from being a vital component of the plant defense, SA also play a pivotal part in DNA damage repair, seed germination, thermogenesis, and abiotic stress tolerance (Dempsey and Klessig, 2017).

The impact of salicylic acid on root-knot nematode pathogenicity in tuberose has not yet been documented in existing literature. Here, we aimed to evaluate the efficacy of salicylic acid in

tuberose by soaking tubers, foliar spray or combined soaking and foliar spray to induce resistance against *M. incognita*.

Material and methods

Plant material: Tuberose tubers cultivar Prajwal (BCKV, West Bengal, India) used as plant material was confirmed as susceptible by inoculation tests (data not shown). Tubers were thoroughly washed under running tap water to remove the soil traces and with tween 20, followed by washes with distilled water until the detergent froth was completely washed. Then the tubers were treated with 0.2 % Bavistin (Carbendazim 50 % WP) overnight to ensure the removal of any fungal contamination. The plants growth was maintained in controlled chambers with a 16-h/8-h light/dark cycle at 22 °C.

Nematode culturing: Egg masses of root knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood race 1 were handpicked using sterile forceps from the two months post infected susceptible brinjal roots under the stereoscope. The collected egg masses were incubated (26±2 °C) for hatching on the double layered tissue paper placed on a molded wire mesh sieve (25 µm) in a glass petri dish containing distilled water. The freshly hatched nematodes (J2) traversed the soft tissue to the water surface. The J2 suspension was then collected using a micropipette under the stereoscope and concentrated to small volumes of sterile water by centrifugation for 5 min at 5000 rpm. This method of nematode hatching is also known as the decanting and sieving method which was originally described by Cobb (1918).

Treatment with Salicylic Acid: Tuberose tubers in a group of forty (10 tubers per concentration) were treated with different SA concentrations (2.5 mM, 5 mM, and 10 mM) according to the following procedure: (T1 treatment) Tubers were soaked in SA solution overnight and in water for control. Next day, these were blot dried for 1 hr and transferred to 6 inch pots containing sterilized soil rite mix. (T2 treatment) Tubers were grown for 3 weeks in sterilized soil rite and the SA solution was applied on plants by foliar spray (covering the soil surface underneath) until dripping using a spray applicator for three consecutive days and those sprayed. Control plants were sprayed with distilled water. (T3 treatment) In this method, the SA soaked tubers were allowed to grow for 3 weeks in soil rite and the plants were then applied SA foliar spray for three consecutive days. For all the treatments the four week old plants were inoculated with 500 J2/plant by pipetting 3 mL of J2 stirring suspensions into 3 holes made in soil around the plant roots. A completely randomized block design was used for the experiment.

Plant fitness assessment: The tuberose tubers with complete roots were carefully removed from the soil after 45 days post inoculation (DPI) for each treatment. These were thoroughly washed under the running water stream to remove the surface soil dirt and softly dried on tissue paper. The data on plant growth variables were measured (e.g. tuber weight (TW, g), root weight, (RW, g), shoot weight (SW, g), number of leaves (L) and shoot length (SL)). Shoot length was measured from the ground level to the apical meristem of the longest leaf.

Root architecture analysis: The roots of each tuberose plant were scanned using root scanner (LA 2400, REGENT

INSTRUMENTS Inc.) and the root image was analyzed for various root characteristics like root length (RL, cm), root volume (RV, cm³), root projected area (RPA, cm²), average root diameter (AD, mm), root surface area (RSA, cm²), numbers of forks, tips and crossing in WinRHIZO software (Arsenault *et al.*, 1995). Root morphological characteristics were measured based on Regent's non-statistical method with overlap compensation.

Crop performance evaluation: Tuberose resistance to *M. incognita* was evaluated based on the root knot index after 45 DPI. For this, the roots were bleached for 5 min with sodium hypochlorite (NaOCl) and boiled for 1 min in acid fuchsin stain (Bybd *et al.*, 1983). The roots were washed in distilled water and destained using glycerol. Visual examination of the root galls was performed to record the number of root galls, females, egg masses and eggs per egg masses for each root system under the stereomicroscope. Additionally, nematode multiplication factor (MF) was calculated for each treatment as [(number of egg masses × number of eggs per egg mass) ÷ nematode inoculum level] (Dutta *et al.*, 2015).

Statistical Analysis: The data were subjected to analysis of variance (one way ANOVA) to evaluate the impact of the treatments on all parameters and means were compared with Tukey's test ($P < 0.05$) using SAS 9.3 (SAS Institute Inc., Cary, North Carolina, United States).

Results

Effect of different salicylic acid treatment on tuberose growth parameters: Data was obtained for the different plant growth parameters including shoot length (SL), shoot weight (SW), number of leaves (L), tuber weight (TW) and root weight (RW) after 45 DAI (days after inoculation) to assess the effect of SA treatment on the tuberose growth (Fig. 1). SA treated plants did not show any toxic effect, in the form of wilting or yellowing of leaves within the growth period of these 45 DAI. There were no significant differences in shoot weight, number of leaves, and root weight observed among the various concentrations of SA-treated plants compared to the untreated control plants. The shoot length of plants treated with 5 mM SA showed a significant reduction ($P < 0.05$) in T1 and T2 treatments, with recorded lengths of 45.8 cm and 41.5 cm, respectively, compared to the untreated plants with lengths of 51.8 cm and 45.8 cm, respectively. However, no significant differences in shoot length were observed for plants treated with 2.5 mM (T1, T2 and T3), 5 mM (T3), and 10 mM SA (T1, T2 and T3) compared to the untreated control plants. Plants treated with 10 mM SA exhibited significantly higher tuber weight in T1 and T3, while no significant variation in tuber weight was observed in any of the SA concentrations in the T2 method, as well as for plants treated with 2.5 mM and 5 mM SA in all treatments, compared to the control untreated plants.

Effect of SA treatment on the root structure: The root image analysis of the tuberose plants treated with SA (T1, T2, and T3) did not reveal any significant negative impact on root growth compared to the control (Fig. 2). In the case of tuberose plants subjected to T1 treatment (tubers soaked in SA solution), a significantly positive effect of SA was observed in root growth parameters, such as total root length (RL), tips, forks, and crossings, using WinRHIZO software, in comparison to untreated (UT) control plants (Fig. 3a-c). Although several other root

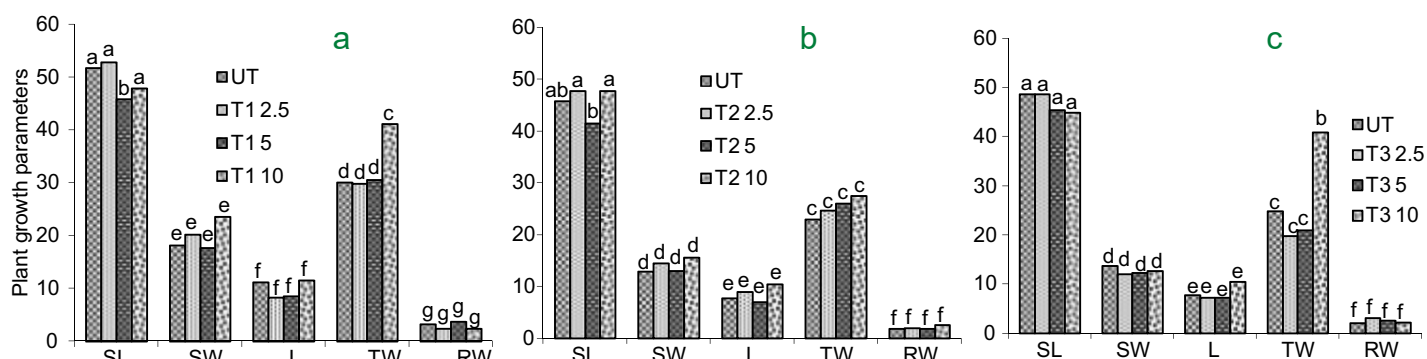


Fig. 1. Effect of different treatment of salicylic acid at 2.5 mM, 5 mM and 10 mM concentration on tuberose growth parameters: Shoot length (SL, cm), Shoot weight (SW, g), Number of Leaves (L), Tuber weight (TW, g) and root weight (RW). (a) T1 treatment: soaking of tubers in SA solution (b) T2 treatment: Foliar spray of SA solution (c) T3 treatment: soaking of tubers along with foliar spray. Data are mean \pm standard error of four replicates. The bars denoted with different alphabets are significant at $P < 0.05$.

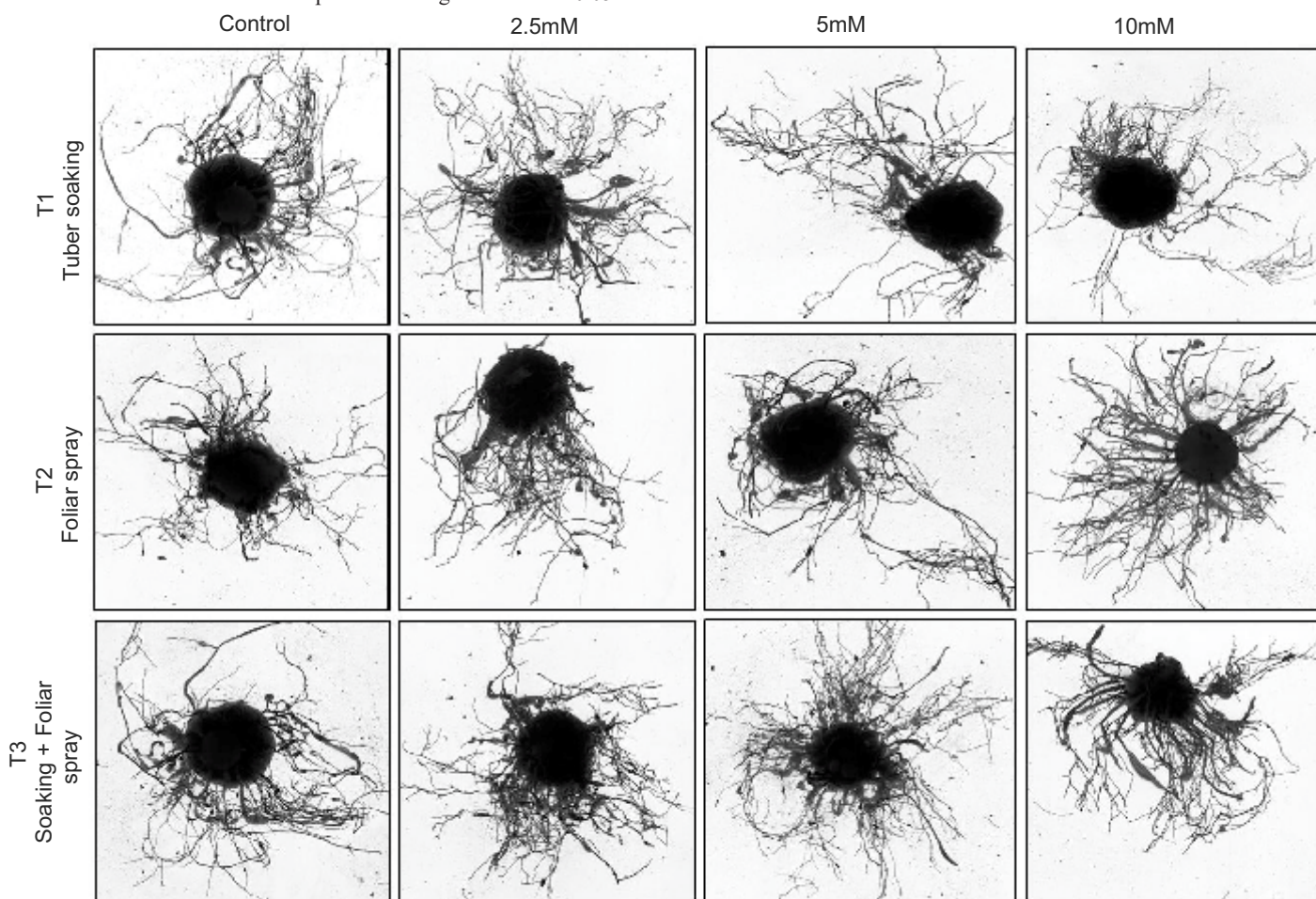


Fig. 2. Effect of different treatment of salicylic acid at 2.5 mM, 5 mM and 10 mM concentration on tuberose root growth morphology as observed by root scan analysis using WinRHIZO software

growth parameters, including projected area (RPA), surface area (RSA), average diameter (RAD), and root volume (RL), increased in T1-treated tuberose plants, the differences were not statistically significant ($P < 0.05$) compared to the control plants. The maximum RL was observed for T1 5 mM SA and T1 10 mM SA-treated plants, measuring 481.3 cm and 507.3 cm, respectively, compared to untreated plants (298.7 cm). Additionally, the number of root tips, forks, and crossings were significantly higher for T1 10 mM, with values of 2772, 3503, and 363, respectively, compared to untreated plants with 1635.7, 1573.7, and 167, respectively.

We observed a similar trend for the T2 treatment (Foliar spray

of SA). The plants root growth parameters positively increased for RL, RV, tips, forks and crossing upon treatment with SA via foliar spray (Fig. 3d-f). Root growth parameters such as RPA, RSA, and RAD did not exhibit any significant differences in the T2 treatment across various SA concentrations compared to the untreated control plants. The significant increases in RL (341.6 cm), RV (4.08 cm³), tips (2498.5), forks (2112), and crossings (161.3) were observed only in T2 10 mM compared to the untreated values of 305 cm, 2.69 cm³, 2025, 1306.8, and 79.3, respectively. The insignificant increases in RL, RV, tips, forks, and crossings were also noted for T2 2.5 mM and T2 5 mM compared to the untreated control.

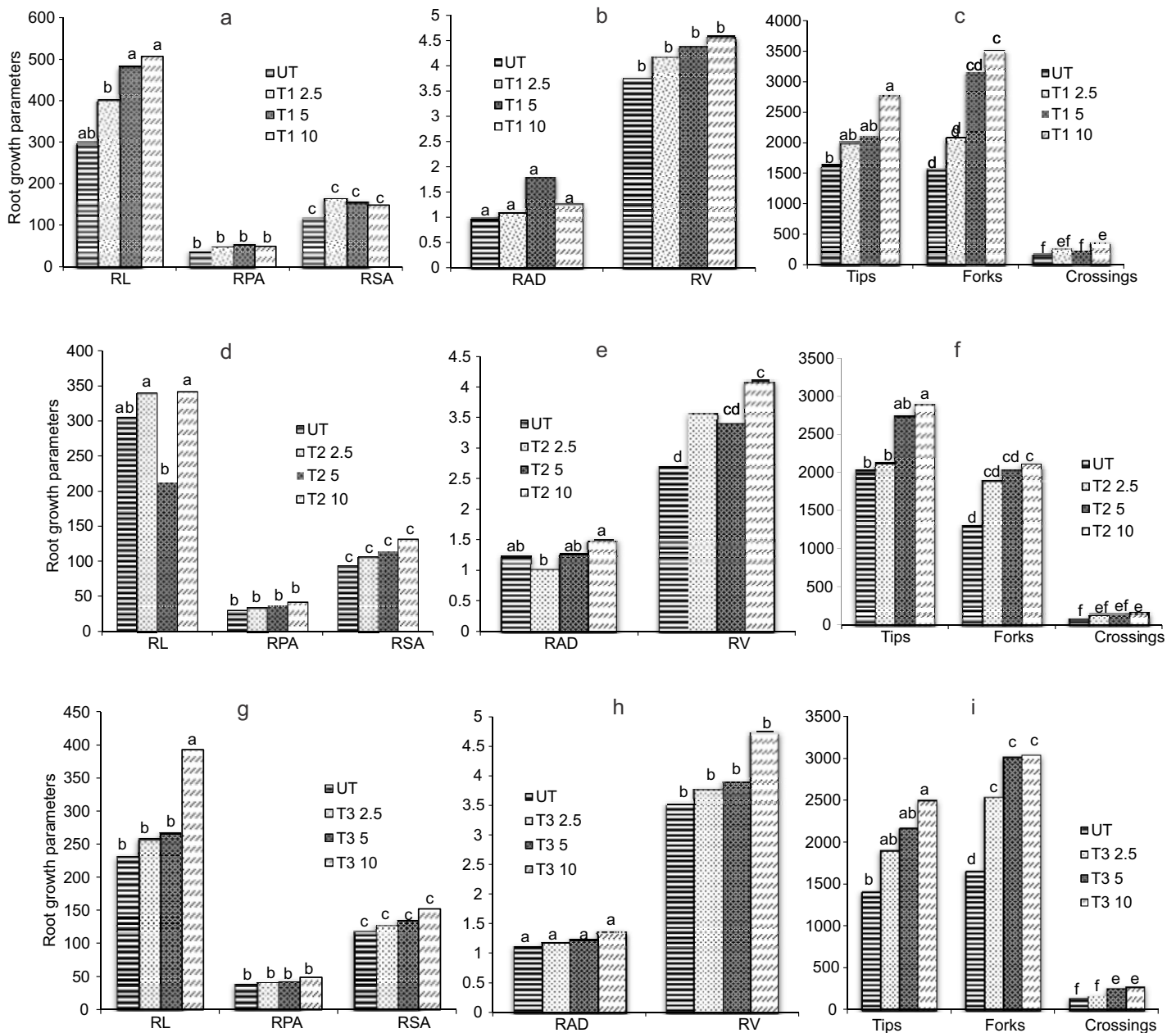


Fig. 3. Effect of different treatment of salicylic acid at 2.5 mM, 5 mM and 10 mM concentration on tuberose growth parameters: Total root length (RL, cm), Root projected area (RPA, cm²), Root surface area (RSA, cm²), Root average diameter (RAD, mm), Root volume (RV, cm³), Number of Tips, Forks and Crossings T1 (a-c) treatment: soaking of tubers in SA solution T2 (d-e) treatment: Foliar spray of SA solution T3 treatment (g-h): soaking of tubers along with foliar spray. Data are mean \pm standard error of four replicates. The bars denoted with different alphabets are significant at $P < 0.05$.

In case of the T3 treatment, root scanning revealed a significant increase in RL, tips, forks, and crossings at the 10 mM SA concentration compared to the untreated control (Fig. 3g-h). Insignificant differences were recorded for parameters like RPA, RSA, RAD, and RV for all T3-treated plants. RL and the number of tips for T3 2.5 mM and T3 5 mM treated plants were at par with the untreated control plants (230.3 cm and 1407.5), while a significant increase was observed for T3 10 mM treatment, with RL (392.6 cm) and tips (2498.2). There was a steady increase in the number of forks with the application of increasing concentrations of SA, ranging from 2526.8 to 3031.5 compared to the control (1653.5). The number of crossings significantly increased in T3 5 mM (254.8) and T3 10 mM (274.3) compared to the untreated plants (138.3).

Effect of different treatments of SA on *M. incognita* reproductive parameters: To assess whether the application

of exogenous SA via different treatment methods was able to induce resistance to *M. incognita*, tuberose plants were inoculated with 500 juveniles of *M. incognita* per plant. After 45 DAI, the obtained data indicated that all the inoculated treatments showed variable significant decreases in *M. incognita* infection in terms of number of galls, females and eggmasses per plant as compared to the inoculated untreated control (Fig. 4). The reduction in number of galls, females and eggmasses decreased gradually with the increasing concentration of SA and the maximum reduction was observed for 10 mM SA in all the different methods of SA treatment compared to untreated control. The average number of galls per plant was significantly reduced ($P < 0.05$) by 36.2 %, 37.8 % and 44.5 % in 2.5 mM SA, 5 mM SA and 10 mM SA in T1 treatment respectively compared to untreated plants. Accordingly, the number of females was reduced significantly in T1 treatment and the percentage reduction was observed as 62.1

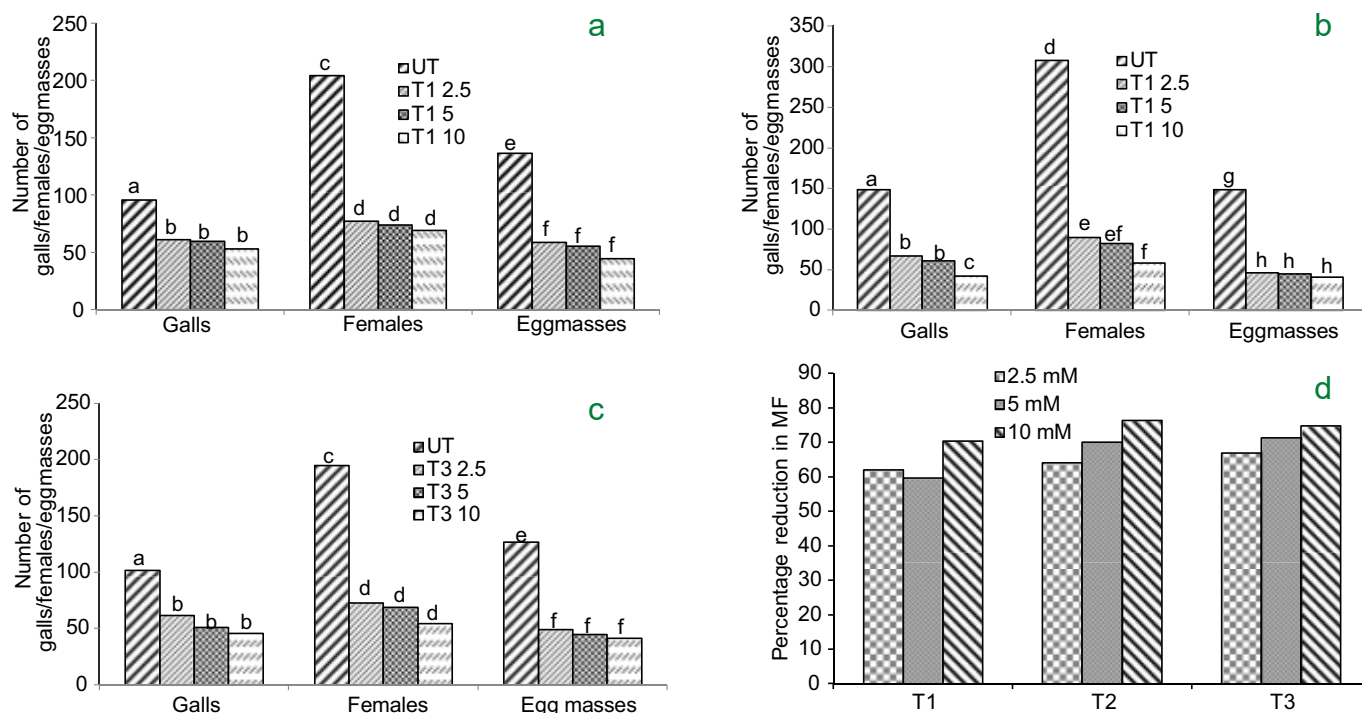


Fig. 4. Effect of different treatment of salicylic acid at 2.5 mM, 5 mM and 10 mM concentration on the number of galls, females and egg masses per plant in tuberose inoculated with *Meioidogyne incognita* (a) T1 treatment: soaking of tubers in SA solution (b) T2 treatment: Foliar spray of SA solution (c) T3 treatment: soaking of tubers along with foliar spray (d) Percentage reduction in nematode multiplication factor (MF) in T1, T2 and T3. Data are mean \pm standard error of four replicates. The bars denoted with different alphabets are significant at $P < 0.05$.

%, 63.8 % and 66.1% for 2.5 mM SA, 5 mM SA and 10 mM SA respectively compared to untreated (Fig. 4a). Comparable results were obtained when the reproduction potential of nematodes was measured as the number of egg masses showed significant reductions of 58.6 %, 60.0 % and 67.3 % ($P < 0.05$) compared to untreated control, respectively (Fig. 4a). Similarly, the average number of galls per plant reduced by 54.8 %, 58.8 % and 71.6 % in T2 treatment with 2.5 mM SA, 5 mM SA and 10 mM SA respectively as compared to the untreated control (Fig. 4b).

The number of female endoparasites reduced by 70.9 % to 80.9 % in T2 treatment compared to untreated control. The SA treatment showed significant reduction of 66.8%, 67.8%, and 72.6% in the number of egg masses per plant for the T2 treatment, using 2.5 mM SA, 5 mM SA, and 10 mM SA, respectively, compared to the untreated plants (Fig. 4b). Similar trend was observed when the combined method of soaking tubers along with foliar spray of SA (T3) was employed (Fig. 4c). The *M. incognita* infection reduced significantly with the application of different concentrations of SA. The percentage reduction in number of galls, females and egg masses ranged from 39.2 % to 54.9 %, 62.6 % to 71.9 % and 60.9 % to 67.3 % respectively, in the T3 treatment compared to the untreated tuberose plants.

The T1 treatment with 2.5 mM SA exhibited the least significant reduction in the number of galls, females, and egg masses, with values of 36 %, 62 %, and 58 %, respectively. In contrast, the T2 treatment with 10 mM SA showed the maximum reduction, with decreases of 72 %, 81 %, and 73 % in galls, females, and egg masses, respectively, compared to untreated tuberose plants (Fig. 4). Notably, the nematode multiplication factor (MF), which determines the reproductive potential and parasitic success of a given nematode, was significantly reduced ($P < 0.05$) by 60 % to 76 % in the SA-treated plants compared to the untreated control

(Fig. 4c). In essence, the exogenous application of SA induces resistance to *M. incognita* by drastically reducing nematode infection, development, and reproductive fitness.

Discussion

Salicylic acid modulates the localized and systemic acquired resistance by accumulating in plants endogenously in response to pathogen attack. Exogenous application of salicylic acid mimics the pathogen attack to induce the expression of defense related genes that are generally switched on when they are invaded by a pathogen (Klessig and Malamy, 1994). SA offers an environmental friendly and sustainable substitute to the routinely used chemical nematicides against the devastating RKNs (Du *et al.*, 2021). Mukherjee *et al.* (2012) reported that the SA treated tomato plants showed reduced nematode infestation in terms of root gall numbers and egg masses along with improved shoot weight. Our study demonstrated the effect of exogenous application of different SA concentrations (2.5 mM, 5 mM and 10 mM) against *M. incognita* in tuberose plants. The findings were in accordance with Molinari *et al.* (2014) that SA application as soil drench restricts the proliferation of nematode juveniles in tomato roots by activating the systemic acquired resistance. Similarly, we found that the external application of SA at 10 mM in the form of tuber soaking, foliar spray or as a combination of both methods suppresses the nematode infection as implicated by the reduction in number of root knots, females and egg masses per plant.

The plant fitness assessment in the present study implied that SA treated plants had no significant differences in the shoot length and weight, number of leaves, tuber weight and root weight for all the SA concentrations. This reflected that the SA concentrations used in this study were not toxic to the plants. Moreover, the root image analysis manifested the positive impact of 10 mM SA on the root length, number of root tips, forks and crossing

compared to the *M. incognita* inoculated control plants. Although the growth was not positively stimulated in 2.5 mM and 5 mM SA treatment, the plants remained healthy and did not show any toxic effect. Nevertheless, applying 10 mM SA was found to be optimal for the beneficial effect on plant growth parameters. It is speculated that perhaps the root growth enhancing effect evident in 10 mM SA treatment is due the reduction in *M. incognita* pathogenicity.

As previously documented, the use of SA as a foliar spray not only promoted the plant development but also induced resistance to *M. incognita* (Nandi *et al.*, 2000; Molinari 2002). However the effectiveness of SA treatment can be dependent on several factors, therefore the present investigation optimized the experimental protocol as well as ideal dosage for the SA treatment to achieve the maximum resistance against the RKN in tuberose. The treatments used in the study to elicit the plants against *M. incognita* did not produce any harmful effects on its growth while simultaneously reducing the nematode infection. Regardless of the method of application, 10 mM SA proved to be ideal for treating the plants. Additionally, significant difference found in percentage reduction of nematode multiplication factor thereby it is evident that 10 mM SA treatment either as foliar spray or in combination with soaking of tubers has potential to reduce *M. incognita* infection. For its simplicity of application, foliar spray is the ideal approach to resist *M. incognita* infection in tuberose. Though, SA role against RKN has been well documented in several crops, this is the first evidence of SA-induce resistance in tuberose against *M. incognita*. Furthermore, unlike the nematicides, SA approach is safe, sustainable and environmental friendly. Farmers can easily adapt this technique to prevent nematode infection in tuberose fields as foliar spray treatment seems effective and rather easy method to execute.

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