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Collective effect of host delivered RNAi splicing factor and integrase gene on the reproductive potential of root-knot nematode (*Meloidogyne incognita*)

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Abstract
The southern root-knot nematode, *Meloidogyne incognita* is the most widespread and economically important plant parasitic nematode. This obligate parasite is globally distributed can be found in different type of soils. Being polyphagous they have a host range of more than 3000 plant species. Plants affected by them shows both above and below ground symptoms. Yellowing, wilting, stunting are the above ground symptoms whereas galling on root is the typical below ground symptom. *M. incognita* is major plant parasitic nematode and it does share a massive contribution in crop losses. Resistance development in host plants is an effective way of nematode control and it can be achieved by RNA interference (RNAi) which refers to development of immunity or resistance in host through post-transcriptional gene silencing of specific nematode genes. With this regard research was conducted to observe of effect of combinatorial host induced gene silencing (HIGs) on the reproductive potential of *M. incognita* on Arabidopsis. Splicing factor and integrase gene were targeted for silencing in *M. incognita* resulted in a decrement of egg mass from 80.27% to 82.18% as compared to the untransformed plants and with reduction in egg per egg mass by 70.27% as compared to the untransformed event.

Keywords: RKN, *Meloidogyne incognita*, HIGs, RNAi, dsRNA

Introduction
Root knot nematode is the most devastating group of nematodes affecting the yield and quality of agricultural and horticultural produce. Worldwide, plant parasitic nematodes account for $173 million loss annually (Elling *et al*., 2013) [1]. Their cosmopolitan nature and complex strategy of parasitism make them distinguished parasites. RKNs are sedentary endoparasites with sedentary melon like females and vermiform males, 2nd juvenile stage is the infective stage which wanders in soil after the hatching in search of the host roots, and they get attracted to the zone of elongation, where they penetrate the root and then migrate intercellularly and separating cells at the middle lamella in the cortical tissue. This process appears to include both mechanical force and enzymatic secretions from the nematode (Atkinson *et al*., 1988; Li *et al*., 2011) [12, 14]. The infective juveniles usually migrate down to the root tip, then turn around in the region of the root apical meristem. They then migrate up the centre of the root to the zone of differentiation establish a feeding site, RKNs induce morphological and anatomical change in host roots with the help of certain oesophageal secretions called as effectors (Bird, 1968) [2]. Followed by subsequent moulting some develops to male and wander in soil and the some develops in female, which remains inside the roots and maintains the feeding site (Giant cell) and draws nutrition from it, Giant cells have dense cytoplasm and thickened walls remodelled to form elaborate ingrowths, much like syncytial cells formed by cyst nematodes. The multiple nuclei in giant cells result from mitosis uncoupled from cytokinesis. In addition, individual nuclei have a high DNA content (Wiggers *et al*., 1990) [3]. Female after maturation lays egg masses in a gelatinous matrix through the secretions of the rectal glands. The infected plants remain stunted, show wilting symptoms and become prone to enhanced susceptibility to other diseases (Fairbairn *et al*., 2007) [13].
RNA interference (RNAi) is a conserved phenomenon of post transcriptional gene silencing through which explicit genes can be silenced in eukaryotes which may govern the vital activities in a target organism. It was first described in *Caenorhabtidis elegans* (Fire *et al*., 1998) [15], this highly conserved mechanism of RNAi has been demonstrated in various organisms belonging to different species across the animal and plant kingdoms (Jones *et al*., 2011) [16].

RNAi is being used as an effective method for gene function analysis and engineering of resistance in the different plant against various pathogens (Lindbo *et al*., 2005) [4]. In the process of RNAi, an exogenous dsRNA is introduced into the eukaryotic organism, the RNAi pathway is started by the RNAase III enzyme which is also called dicer enzyme, Dicer is joined with RNA-binding proteins, the TAR-RNA-binding protein (TRBP), PACT and Ago-2 (Lee *et al*., 2006) [5] which disintegrate the dsRNA in 21-25 small nucleotides sequence called small interfering RNA (siRNA) (Elbashir *et al*., 2001) [6]. The siRNA incorporate into the protein complex (RISC: RNA induced silencing complex), afterward, the RISC is guided to the targeted mRNA and the siRNA complementary to the mRNA starts degrading it. The target recognition and the cleavage is achieved with the help of the argonaute protein in the RISC (Hammond *et al*., 2001) [7].

The most imperative aspect of RNA interference is that, it is highly precise, remarkably powerful and the interference can be caused in cells and tissues far away from the site of introduction (Rosso *et al*., 2009; Tomoyasu *et al*., 2008) [17, 18].

Host generated RNAi is a ground-breaking approach for the delivery of dsRNAs or siRNAs into the feeding nematodes for the silencing of vital nematode specific genes. A dsRNA construct for the target gene is developed by cloning a part of the target gene cDNA in sense and antisense orientation separated by an intron or spacer region. A strong tissue specific or constitutive promoter may be used to drive the expression of the dsRNA. Transcription of the sense and antisense strands results in the formation of a self-complimentary hairpin structure with the removal of the intron by splicing (Smith *et al*., 2000) [19]. The dsRNAs so formed can either be directly ingested by the PPNs or can be processed by the host plant's own RNAi machinery and the resulting siRNAs can be subsequently ingested by the PPNs (Bakhetia *et al*., 2005; Dutta *et al*., 2015) [21].

Material and Methods

The research was conducted in the Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi. The seeds were surfaced sterilized in the laminar air flow chamber with the help of sterilizing agent SDS (0.1%) mercuric chloride (0.1%) and ethanol (70%). The seeds were given first wash with DDW autoclaved water and were then vortexed for a minute with a subsequent centrifugation for 1 minute at the rate 1000rpm. Keeping the same conditions of vortexing and centrifugation the seeds were then washed with SDS+ mercuric chloride, then with ethanol and finally 5 washes of DDW autoclaved water were given.

The seeds after being kept at 4 °C for 48 hours were taken to spread over the freshly prepares agar gel Petri plates (Fig2a). The seeds were carefully placed on the agar gel with the help of autoclaved micro tips. The Petri plates were then sealed with parafilm and the plates were kept in the culture room by maintaining a temperature of 22 °C and 16 hours of continue light and 8 hours of darkness.

The root-knot nematode (*Meloidogyne incognita*) population was taken from the culture plants maintained by our laboratory at the Division of Nematology, IARI, New Delhi. The tomato plants were uprooted, roots washed with double distilled water until absolutely clean and egg masses handpicked in a cavity block. Egg masses were surface sterilized with 0.1% $HgCl_2$ for one minute and then rinsed with sterile distilled water three times so as to wash off the surface sterilizing agent. The egg masses were placed in the BOD for hatching at 26-28 ºC. The freshly hatched infective juveniles were collected in a beaker in suspension and were counted and calibrated per ml. An average of 3 aliquots was taken and used further inoculums. 500 IJs were inoculated per plant. (Fig2b). The egg masses laid by *M. incognita* females were isolated from T_1 and control tomato plants and their number was counted per plant to see the difference between fecundity. The gelatinous matrix was dissolved by rinsing the egg masses with 0.5% NaOCl in an effendorf tube for 3 min (Hussey *et al*., 1973) [8]. The eggs were counted under the stereomicroscope using counting dish. 5 replication were taken for each treatment. The data taken from every replicate of each treatment were analysed for variance (ANOVA) and CRD test was done to verify the data as significant or non-significant. The means were calculated by using Tukey's test at the $p<0.01$ significance level using software, SAS for Windows (V 9.3 Chicago, USA).

Result

The effect of combinatorial host delivered silencing of nematode genes on the reproductive potential of *Meloidogyne incognita*. The egg masses were counted and the Arabidopsis plants modified for individual gene HIGs were noted to bear significantly less egg mass as compared to the untransformed ones (Fig 3). The crossed Arabidopsis plants resulting in HIGs of both the genes showed a significant reduction in the number of egg mass ranging with a decrement of egg mass from 80.27% to 82.18% as compared to the untransformed plants (Table 1) (Fig1).

Stereomicroscope was used to determine the number of eggs per egg mass (Fig 3). The recorded data showed that the individual RNAi lines of Arabidopsis for genes *splicing factor* and *integrase* had reduced eggs per egg masses. In the events silenced for the gene-splicing factor the eggs per egg mass was reduced by 60.70% and that of *integrase* gene by 45.38% and the crossed lines expressing the dsRNA of both the gene showed reduced eggs per masses as compared to the individual events RNAi silenced for each gene. Out of the 4 crossed events the most significant was SFIT2 with reduction in egg per egg mass by 70.27% as compared to the untransformed event (Table 1) (Fig 1).

Table 1: Variation of reproductive capability of *Meloidogyne incognita* in different treatments

Independent Crossed Lines	No. of Egg mass	% Decrease	Number of Eggs/Egg mass	Percentage Decrease
SFIT 1	5.85	80.76	115.42	70.16
SFIT 2	5.42	82.18	115.00	70.27
SFIT 3	6.00	80.27	126.42	67.32
SFIT 4	5.57	81.68	127.14	67.13
SPL	7.71	74.3	152.00	60.70
INT	12.57	58.6	211.28	45.38
Untransformed	30.42	0	386.85	0

CD (P=.05)	1.258		9.692	
F (CAL)	429.62		845.65	
F (Table) at 1%	3.26			

Significant Values at P= 0.01
Data: Average of 5 Replication

Discussion

Meloidogyne incognita is a major plant parasitic nematode with wide host rang and high damage potential. RNAi has been a used method to silencing the pathogenic gene, housekeeping gene as well as reproductive genes through in vitro methods and HIGs. In our study we observed the silencing effect of two combined genes in *M. incognita* through HIGs on reproductive capability of *M. incognita*.

In the study the combined silencing of integrase and splicing gene (SFIT1, SFIT2, SFIT3 and SFIT4) showed significant reduction in the egg mass of *M. incognita* as compared to individual silencing by integrase gene (INT) and splicing gene (SPL). With respect to untransformed plant a reduction of 80.27% to 82.18% was observed in egg mass production. Same pattern was observed in number of egg/egg mass but not up to same extent. Combined gene silencing was more effective than individual silencing. Combined gene silencing showed as reduction of eggs/egg mass 68-70% as compared to untransformed Arabidopsis plants.

Integrase and splicing factor are the housekeeping genes of *M. incognita*. These are involved in splicing of mRNA before translation. Splicing factor genes are known to play an important role in regulating gene expression and alternative splicing in eukaryotic organisms. In *M. incognita*, several splicing factor genes have been identified, including serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). Zhang *et al*. (2019) [9] found that the knockdown of the *M. incognita* SR protein gene Mi-SRP-4 resulted in decreased levels of gene expression and reduced pathogenicity in tomato plants. Furthermore, another study by Li *et al*. (2020) [10] demonstrated that the downregulation of an hnRNP gene, MiRNP-1, resulted in reduced *M. incognita* egg production and altered splicing patterns of several genes involved in the nematode's development and reproduction. In *M. incognita*, the integrase gene has been shown to be highly expressed in the early stages of the nematode's development. It is possible that the integrase gene plays a role in the regulation of gene expression and alternative splicing in the nematode's genome during its development. Xu *et al*. (2019) showed that the knockdown of the integrase gene in *M. incognita* resulted in a significant reduction in the nematode's reproductive capacity and pathogenicity towards tomato plants. This suggests that the integrase gene may also play a role in the nematode's pathogenicity and reproductive success. The individual effect of these genes on fecundity of *Meloidogyne incognita* is significant, the silencing of integrase gene is observed to be more effective as compared to silencing of splicing factor gene. However, the effect of combined silencing effect of both genes is far better than their individual silencing on reproductive capability of *M. incognita*. There must be certain interdependence on the gene product of these two genes, combined silencing of above genes have synergistic effect. More studies are required to study the proteins related to the genes and their function in order to relate their interdependence.

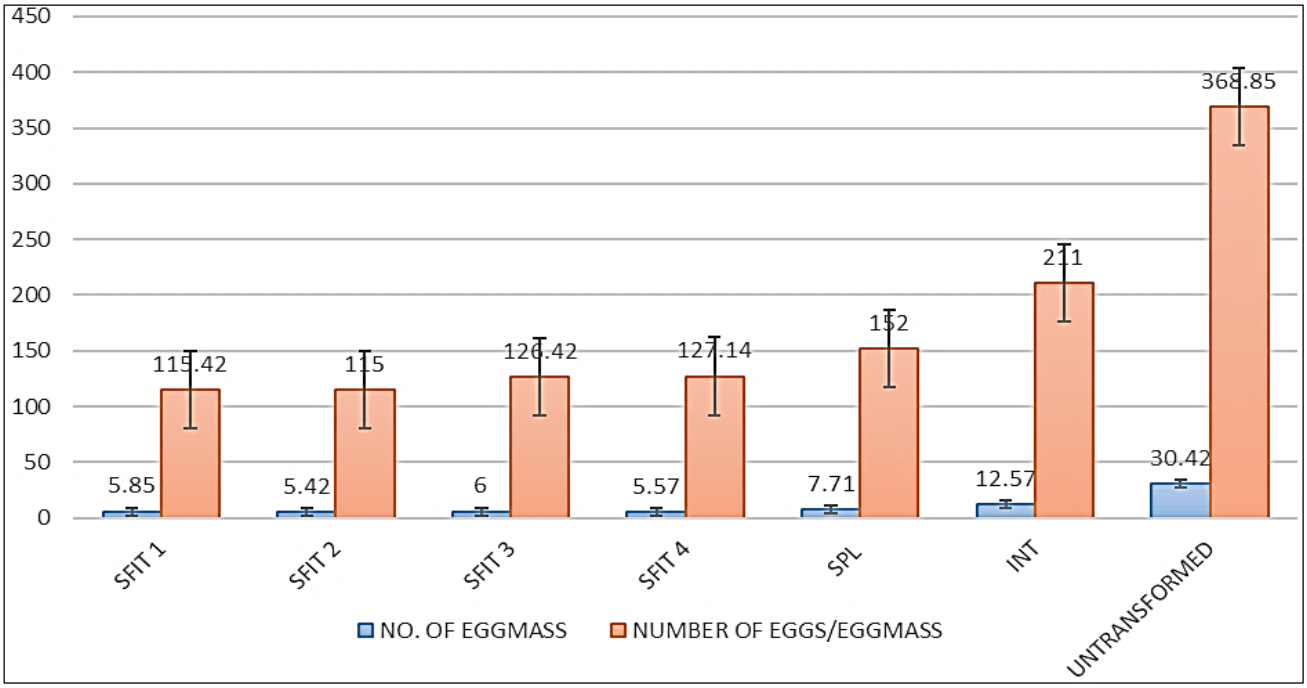

Fig 1: Variation of reproductive capability of *Meloidogyne incognita* in different treatments

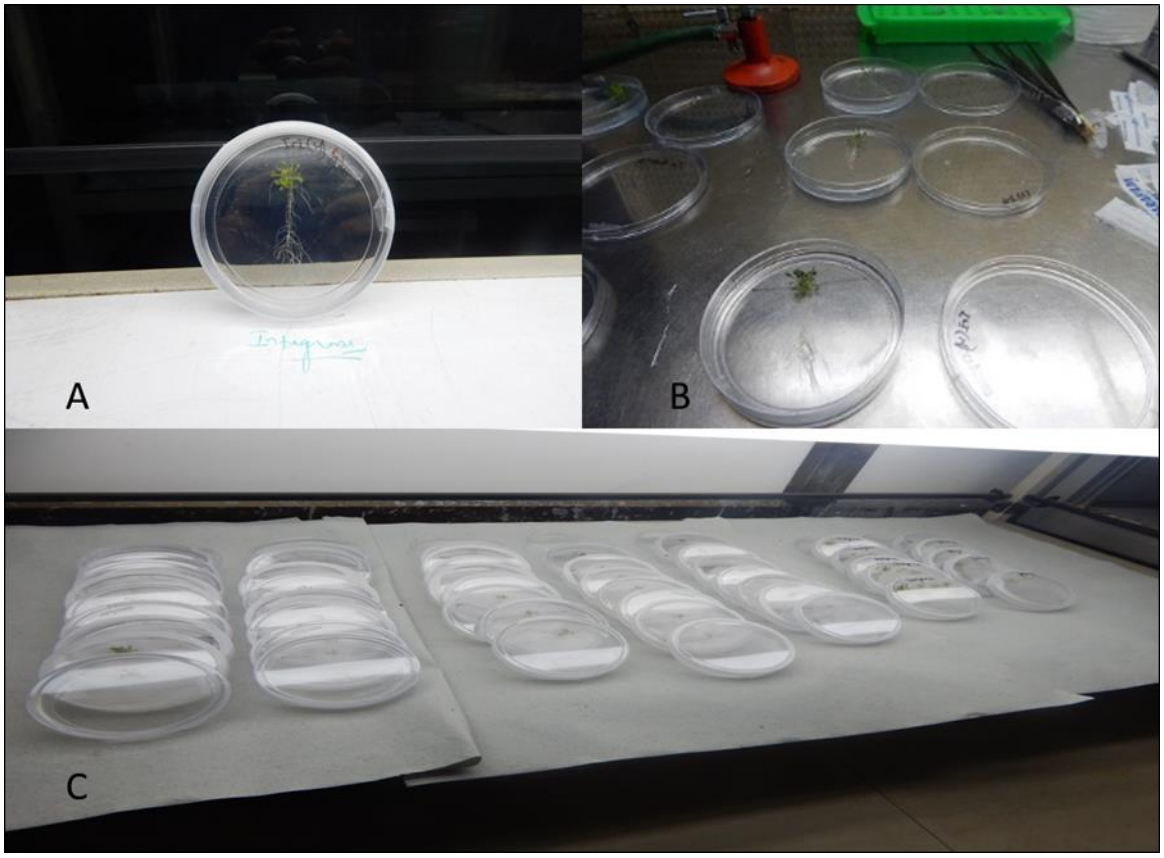

Fig 2: Different step in progress of experiment showing (a) individual plant grown in Half MS media with clerigel (b) Inoculation of juvenile of *M. incognita* to different treatment at the rate 500IJs/ plant in laminar air flow (c) different treatments shown with their replication

Fig 3: Observation of egg mass and egg/egg mass under microscope

Conclusion

The HIGs in Arabidopsis resulted in decreased fecundity of *Meloidogyne incognita*. The combined silencing of integrase and splicing factor gene resulted in a decrement of egg mass from 80.27% to 82.18% as compared to the untransformed plants and with reduction in egg per egg mass by 70.27% as compared to the untransformed event.

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