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Integration of biocontrol agents with fungicide, weedicide and plant growth regulator for management of stem and root rot of jute

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Abstract: Combination of chemical fungicides (*viz.,* Carbendazim 50 WP and Tebuconazole 250 EC) and biocontrol agents (*viz., Pseudomonas fluorescens* Psfl1, *P. striata* Pst1, *Azotobacter chrococcum* Azbc3, *Bradyrhizobium japonicum* Brj4, *Trichoderma aureoviridae* S12, *T. harzianum* JTV2, *T. virens* JPG1, *Aspergillus niger* AN15 strains respectively either singly or in consortium) were used to counteract *Macrophomina phaseolina,* the causal organism of stem and root rot of jute. In addition, suitable plant growth regulator *viz.,* Indole-3-acetic acid (100-1.0 µg/ppm) and herbicide Quizalofop ethyl 5 % EC were used to augment the activity of *Trichoderma. T. aureoviridae* strain S12 was found to be the best among the eight isolates screened for tolerance against the two fungicides and herbicide at a concentration of 10000 - 500 µg respectively as well as against *M. phaseolina* (Inhibition=72.33 %) *in-vitro.* This strain showed best compatibility with other strains and highest tolerance to fungicide *i.e.,* Carbendazim 50 % (up to 500 µg). Highest number (13.7×10^6) of active spores was recorded at a concentration of 25 ppm of IAA under *in-vitro* condition. S12 recorded a biocontrol efficiency of 61.8 % against stem rot of jute along with significant plant growth promotion and fibre production. Plant biomass also increased up to 7.5-12.1 % and fibre production 37.0-39.9 % with fungal and bacterial consortium + carbendazim seed dressing and soil drenching. These biocontrol fungi and PGPR consortium with high tolerance to fungicide, weedicide and plant growth regulator up to certain extent may be potentially exploited in IDM which may be a low cost technology in jute and allied fibre crops.

Keywords: Biocontrol, Fungicide, Growth regulator, *Macrophomina phaseolina, Trichoderma*, Weedicide

INTRODUCTION

In recent times, to satisfy the demand of ever-increasing global population, chemical fertilizers and pesticides are indispensable in modern agriculture for achieving higher yield of crops Nonetheless, indiscriminate uses of chemicals impart hazardous effect on soil-microbe-ecological balance, cause phytotoxicity and lysis of beneficial organisms (Dłużniewska, 2003), leads to alarming resurgence of pesticide resistant mutants of pathogens, consequently results in grave environmental pollution due to residual problem (Sayyed and Patel, 2011). This has diverted the attention of plant pathologists toward alternate methods for the control of plant diseases.

Biological control of phytopathogens is an alternative and attractive preposition as it mimics the natural way of balancing population of living organisms without disturbing the microbial diversity of soil while maintaining exponential population growth of the biocontrol agents as well. Over and above it helps to increase the yield by suppressing pathogen inoculum, protects plants against infection by inducing resistance and all the more enables to attain a safe and clean environment (Bandopadhyay and Bandopadhyay, 2004).

To this end, Plant Growth-Promoting Rhizobacteria (PGPR), Biofertilizers and Biocontrol fungi in consortial assortment has presented immense potential in field evaluation as effective substitute for agrochemicals. Coincidentally, some soil-borne root infecting fungi cannot be eradicated by chemical fungicides because they produce persistent resting structures like sclerotia, pycnidia etc. for their survival for a longer period of time under adverse environmental conditions (Baker and Cook, 1974). Under such circumstances, direct antibiosis and hyper-parasitism exhibited by most biocontrol fungi needs to be exploited simultaneously, integrating with plant growth regulator and herbicide improves yield by triggering active spore germination of biocontrol fungi and increases fungi-

cide tolerance respectively (Adekunle *et al*., 2001). Thus, inclusion of all such components to integrated disease management (IDM) is ultimate practical approach for cost effective sustainable agriculture (Bandopadhyay *et al.,* 2006).

Trichoderma species are known to suppress infection of root by soil-borne pathogens like *M. phaseolina*, *Rhizoctonia solani*, *Fusarium* sp. and *Pythium* sp. on various crops (Ehtesham *et al*., 1990; Bandopadhyay *et al*., 2009). *Trichoderma* also has growth promoting ability that may be in harmony with biological control (Benitez *et al*., 2004; Dubey *et al*., 2007).

The combined use of bio-control agents and chemical pesticides has attracted much attention in order to obtain synergistic effects in the control of soil borne diseases (Locke *et al*., 1985). Reduced amount of fungicide can stress and weaken the pathogen and render its propagules more susceptible to subsequent attack by the antagonist (Hjeljord and Tronsmo, 1998). Srinivas and Ramakrishnan (2002) have reported that integration of bio-control agents with commonly used fungicides showed positive association by reducing the seed infection compared to fungicide and the fungal antagonists individually.

In India and Bangladesh, every year the fiber yield of jute is being reduced by 30% due to stem and root rot disease caused by *Macrophomina phaseolina* (Tassi) Goid, which has continued to be a burning problem over a century (Gupta and Chauhan, 2005). This study was aimed at evaluating the efficacy of the proposed PGPR consortium comprising of *Pseudomonas fluorescens* Psfl1, *P. striata* Pst1 (Phosphobacter), *Azotobacter chrococcum* Azbc3, *Bradyrhizobium japonicum* Brj4 and fungal consortium consisting of *T. harzianum* JTV2, *T. virens* JPG1, *Aspergillus niger* AN15 and *Trichoderma aureoviridae* S12, in conjunction with low concentration of chemical fungicides *viz.,* Carbendazim 50 Wettable Powder and Tebuconazole 250 Emulsifiable Concentration in an integrated management of stem rot of jute.

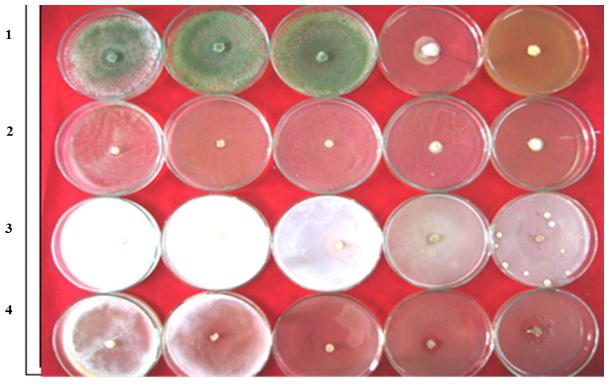

Fig.1. *Differential tolerance of Trichoderma aureoviridae (AB916337) as JTV-1 to fungicide, weedicide and Plant growth regulator chemicals in-vitro.*
***Concentrations Left to right**: 1. Indole-3 Acetic Acid 100, 50, 25, 10, 1 µg (0.01 - 0.0001%) a.i. 2. Carbendazim 10000, 5000, 2500, 1000, 500 µg (1.0, 0.5, 0.25, 0.1, 0.05%) a.i. 3. Tebuconazole 10000 - 500 µg (1.0 - 0.05%) a.i. 4. Quizalofop ethyl 10000 - 500 µg (1.0 - 0.05%) a.i.*

MATERIALS AND METHODS

Microbial cultures: Four wild-type PGPR strains Psfl1 (*Pseudomonas fluorescens*), Pst1 (*P. striata*), Azbc3 (*Azotobacter chrococcum*), Brj4 (*Bradyrhizobium japonicum*) and four PGPF strains JTV2 (*Trichoderma harzianum*), JPG1 (*Gliocladium virens =T. virens*), AN15 (*Aspergillus niger*) and S12 (*T. aureoviridae*) were used in the experiment. These strains were isolated from jute rhizosphere growing in the alluvial tracts of the Ganges, identified by 16S and 18S rDNA sequencing and analyzing the sequence using program (http://www.ddbj.nig.ac.jp). These strains were applied as seed bacterization, culture suspension of spores adjusted to 2×10^5 spores per cm^3.

Macrophomina phaseolina (Tassi) Goid isolated from infected jute stem. Inoculum prepared as 5 mm disc on 60 g sterile discarded jute seeds + 2 % dextrose mix. Inoculum added at a concentration 1.5 g/ kg of soil.

Jute cultivar: *Corchorus olitorius* L. var. JRO 524 (Navin) susceptible to stem and root rot of jute was used in this experiment. Seeds were planted in 30 cm diameter pots filled with non-sterile soil and grown under green- house conditions at 35-37° C, Relative Humidity = 80-90 %.

***In-vitro* evaluation:** *T. aureoviridae* was evaluated for tolerance against fungicides, herbicide and growth regulator before pot trial using food poison method *in-*

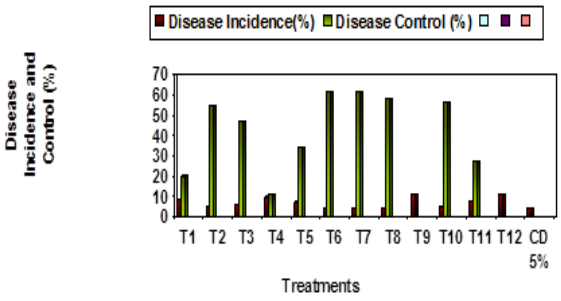

Fig. 2. *Effect of bioformulations on disease incidence and control of Macrophomina.*

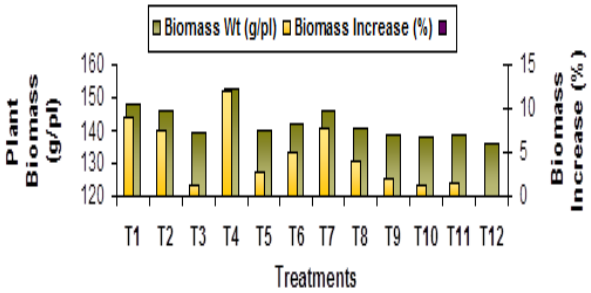

Fig. 3. *Effect of bioformulations on biomass of Macrophomina.*

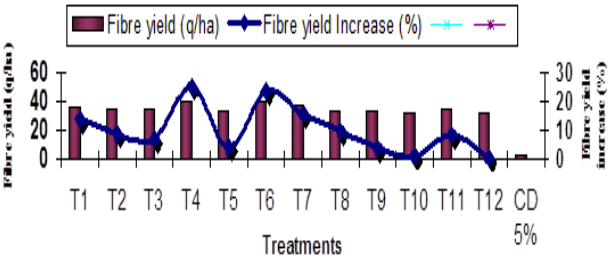

Fig. 4. *Effect of bioformulations on fibre yield of Macrophomina.*

Fig. 5. *Test of Compatibility/Synergistic effect of Trichoderma aureoviridae.*

vitro. Growth regulator *indole-3 acetic acid* or IAA (25 ppm), fungicides *viz.*, Carbendazim 50 WP and Tebuconazole 250 EC and weedicide Quizalofop ethyl 5 % EC were added to Potato Dextrose Agar medium (PDA: Potato Peeled-200 g, Dextrose-20 g, Agar-20 g, water-1 L) to get final concentration of 500, 1000, 2500, 5000 and 10,000 µg for fungicides and weedicide respectively and 1, 10, 25, 50, 100 µg for growth regulator chemicals. Number of spores at different concentration of IAA was checked by *Haemocytometer*. PDA without any chemicals served as control. All the above experiments were carried out in 3 replications. A 5 mm inoculum disc of *Trichoderma aureoviridae* was cut from the margin of actively growing colony and placed in centre of each Petri plate. Petri plates were incubated at 25±2 ºC. Radial growth of *Trichoderma aureoviridae* was observed daily. After 72 hours of incubation the germination process was stopped by adding a drop of formalin. Next a degree of spore's germination was estimated. Percent inhibition of radial growth of the stem rot pathogen offered by all the *Trichoderma* isolates was also checked and confirmed by dual culture before formulation used (Bhattacharyya *et al*., 2014). Biocontrol agents were also screened for fungal cell wall-degrading enzyme (chitinase, β-1,3glucanase), siderophore and HCN production before pot experimentation.

***In-vivo* evaluation:** The evaluation was done after *in-vitro* selection of efficient *Trichoderma* sp. tolerant to agrochemicals and plant growth regulator (Khan and Shahzad, 2007) following the compatible combinations among them (Bandopadhyay and Bandopadhyay, 2004). The bio-formulations with the selected isolates were evaluated in the following manner.

Pot evaluation trial: Effectiveness of commercial Talc (Hi-media) + Fly ash based bio formulations of fungal consortium and Kaolin (Hi-media) based bacterial consortium singly and integrated with fungicides like Carbendazim 50 % (Bavistin) and weedicide Quizalofop ethyl 5 % EC (Targa super) were evaluated for disease control, plant biomass and fibre production in jute.

Bio-formulations used: Three bio pesticide formulations developed with different microbial inoculant-combination, *viz.* 1. Fungal consortium of *Trichoderma aureoviridae* + *Gliocladium virens* (= *T. virens*) as S12 and JTV2 + *Aspergillus niger* (AN15). 2. Bacterial consortium of *Pseudomonas* + *Phosphobacter* + *Azotobacter* Azbc 3. Fungal + Bacterial consortium of (*T. aureoviridae* + *A. niger* =PGPF) + (*Pseudomonas fluorescens* Psfl1+ *Phosphobacter* Pst1 +*Azotobacter* Azbc3= PGPR)

Seed dressing @ 10 g kg^{-1} seed, Soil inoculants @ 1 kg in 300 sq m. = 0.3 hectare area, Fungicide Seed Treatment by Carbendazim 50 % @ 2 g/2ml kg^{-1} seed, Weedicide Spray by Quizalofop ethyl (Targa Super) @ 2.5 ml l^{-1} water, Plant growth regulator 12 hrs seed soaked in *Indole-3-acetic acid* (IAA) @ 25 ppm, Treatment: 12, Replication: 3, Pot Size: 30 cm in diameter, Design: RBD, Fertilizer application: $N_{40}P_{20}K_{0}$.

Treatments

T1 - Fungal Consortium SD
T2 - Bacterial Consortium SD
T3 - *Trichoderma* + Carbendazim SD
T4- Fungal and Bacterial Consortium (1:1) + Tebuconazole SD
T5- Fungal and Bacterial Consortium (1:1) + Carbendazim SD
T6- Fungal Consortium + Carbendazim SD + Fungal Consortium Soil Drench 15 DAS
T7 - Bacterial Consortium+ Carbendazim SD + Bacterial Consortium Soil Drench 15 DAS
T8 - *Trichoderma* formulate SD + Targa Super Spray 21 DAS
T9- Fungal Consortium Seed Dressing + Targa Super Spray 21 DAS
T10- Bacterial Consortium Seed Dressing + Targa Super Spray 21 DAS
T11- Fungal Consortium + Bacterial Consortium Seed Dressing + Targa Super Spray 21 DAS
T12 - Control/Check

SD = Seed dressing, DAS = Day's after Sowing.

RESULTS AND DISCUSSION

Tolerance to agrochemicals, inhibition of pathogen by dual culture plate, *in-vitro* biocontrol potential and identification of the antagonists: *In-vitro* studies revealed that *Trichoderma aureoviridae* remains active up to a concentration of 500 µg/ppm (0.05 % *a.i.*) of fungicide, Carbendazem 50 % even after 72 hours, although the growth of the isolate was relatively slow, time specific and the active spores are expressed in

minute to moderate in number and their proliferation was quite late over the control (Fig. 1). It was observed that all the fungicides and herbicides used weakened antagonistic fungi spores germination. Out of all the fungicidal preparations, the most powerful fungi static effect was observed for Tebuconazole 250 EC, which in the concentration of 500 µg completely inhibited spores germination in all the fungi tested. Also Quizalofop ethyl 5 % EC (Targa Super) herbicides in the dose of 500 µg significantly inhibited the development of germ tubes in the saprophytes researched. However, growth gradually decreases with increase in concentration and completely inhibited at 1000-10000 µg/ppm (0.1-1.0 % *a.i.*). In contrast to this, Fungicide Tebuconazole 250 EC and weedicide Quizalofop ethyl 5 % EC is very toxic and completely suppresses the growth of *T. aureoviridae* even at the lowest concentration. Thus, the level of tolerance of *T. aureoviridae* to the agrochemicals may be rated as Carbendazim > Tebuconazole and Quizalofop ethyl. As time progresses the effect of the chemicals decrease but mycelia and conidia of the selected fungi slowly proliferate up to certain extent. On the other hand, the activity of the spores and their expression in number are increased up to certain extent as the concentration of plant growth regulator (IAA) increases. The activities of this isolate are promising and very effective in disease management and plant biomass production under field condition, the results of the experiments into the effect of selected abiotic factors varied on effectiveness of *Trichoderma* genus fungi when jute seed-dressing was applied singly and in integration (Fig. 2-4) as presented below. The factors applied in the experiment modified the antagonistic effect of *Trichoderma* genus fungi applied in a form of seed dressing. The effect of the factors studied was considerably related to the saprophyte pathogen relationship and the direction of changes depended on the fungus species as well as the factor kind. The inhibition offered by *Trichoderma* isolates and bacterial biocontrol-agents against the pathogen ranged between 68.88-72.33 %. *T. aureoviridae* tested positive for siderophore and HCN production along with fungal cell wall degrading enzymes, chitinase and β-1, 3 glucanase production. The nucleotide sequence of 18S rDNA of *T. aureoviridae* deposited at DDBJ, Japan was assigned the accession number: AB916337.

Disease management: Seed dressing with bio formulations of fungal consortium + Carbendazim, bacterial consortium + carbendazim with bacterial consortium and fungal consortium soil drench 15 DAS (T6 and T7) resulted in controls of pathogen *Macrophomina phaseolina* up to 61.8 %. Whereas, seed dressing alone with *Trichoderma* formulate + Targa super spray 21 DAS (T8) resulted in control of *Macrophomina* stem and root diseases in jute up to 58.1 % and bacterial consortium + Targa super spray 21 DAS (T10) or bacterial consortium seed dressing only (T2) can control 56.3-54.5 % disease over control. *Trichoderma* and Carbendazim seed dressing + Targa super spray 21 DAS (T3) and fungal + bacterial consortium + carbendazim seed dressing (T5) achieved 47.2-34.5 % disease control (Fig. 2).

Plant growth and fibre yield: Plant biomass and fibre production could be best achieved with fungal + bacterial consortium along with Tebuconazole seed dressing (T4) of which biomass increased up to 12.1 % and fibre production 39.9 %. Plant biomass increased up to 9.1 % being followed by fungal consortium seed dressing (T1) In contrast, highest fibre production could be achieved with fungal consortium + carbendazim seed dressing + fungal consortium soil drench 15 DAS (T6). Intermediate results were obtained for bio formulations comprising of bacterial consortium + carbendazim seed dressing + bacterial consortium soil drench 15 DAS (T7) both in case of plant biomass and fibre yield increase. Seed dressed with fungal and bacterial consortium + Tebuconazole (T4) and fungal consortium + carbendazim seed dressing + fungal consortium soil drench 15 DAS (T6) showed highest fibre yield of 39.8 and 39.6 q h^{-1} respectively. For *Trichoderma* seed dressing + Targa Super spray (T8) and fungal + bacterial consortium seed dressing along with Targa super spray (T11) almost 34-35 q ha^{-1} fibre yield of jute was recorded (Fig. 3 and 4).

Stem rot of jute caused by *Macrophomina phaseolina* has been a century old disease affecting the crop globally, especially in US and south-east Asia including Bangladesh and India, whereby decreasing fibre yield by 30 % (Gupta and Chauhan, 2005). Till date no such *Macrophomina*-rot resistant variety of jute has been launched to counteract the pathogen. The little effort that has been invested on biocontrol is restricted to the use of *Trichoderma viride, Aspergillus niger, Pseudomonas fluorescens* and *Azotobacter* sp. respectively (Meena *et al.,* 2014; Roy *et al.,* 2015).

For the first time, this study has exploited both bacterial and fungal consortium in integration with fungicides, Carbendazim 50 % and Tebuconazole 25.9 % w/ w. *Moreover this work has documented the use of a novel fungus, Trichoderma aureoviridae that has exhibited tolerance to fungicide and other chemicals as well as growth regulator, IAA.* The experiments presented showed that higher concentrations of the used chemicals inhibited mycelium growth, weakened sporulation and spore germination of *Trichoderma* isolates.

Trichoderma sp., an endophytic symbiont has long been investigated as a biological control organism against several soil borne pathogens (Benítez *et al.,* 1998; Lorito *et al*., 2010). The mechanism of biocontrol has been largely attributed to the production of allelo-chemicals that include antifungal diffusible and volatile metabolites with antibiotic activity, cell wall degrading enzymes, Fe^{3+} iron chelating molecules, siderophores, besides its positive influence on growth promotion and increased tolerance to various abiotic

stresses is remarkable (Gravel *et al*., 2007). Biocontrol results either due to competition for nutrients and space or as a result of the ability of *Trichoderma* to produce and/or resist metabolites that either impedes spore germination (fungi -stasis), kill the cells (antibiosis) or modify the rhizosphere. Biocontrol may also result from a direct interaction between the pathogen itself and the BCA due to mycoparasitism, which involves physical contact and synthesis of hydrolytic enzymes, toxic compounds and/or antibiotics that act synergistically with the enzymes (Howell, 2002; Djonovic *et al*., 2006).

Simultaneously, *Trichoderma* has been shown to be capable of increasing plant growth and yield. This increases indirectly accounts for the reduction in plant diseases. This fungus could also have the potential to stimulate plant growth independent of any plant disease. Applications of *Trichoderma* in plant production, therefore, can reduce the use of fungicides, growth regulators and labour which eventually will lower the production costs and environmental impact (Pandya and Saraf, 2010).

The resistance to fungicides, herbicide exhibited by our strain, *T. aureoviridae* can be justified by evidence from previous work (Chet *et al.,* 1997), can be accounted for the presence of ABC system of transportation (Harman *et al.,* 2004). However, unlike the previous works, this strain has exhibited a very high tolerance to Carbendazim, *i.e.,* up to 500 µg/ppm, whereas usual range being (1-50 ppm) (Roy *et al.,* 2015). As observed by Ahemad and Khan (2010), likewise this strain also showed an increase in plant growth promoting activity (evidenced from increase in fibre production and biomass increase) with the increase in herbicide dose until the toxic level was reached.

High tolerance of *T. aureoviridae* to growth regulator, IAA, being 25 ppm, can be correlated to the increase in chitinase activity as has been observed by Badri *et al.,* 2007. He opined that most *Trichoderma* strains can tolerate IAA upto 30 ppm. The role of *Trichoderma aureoviridae* is immense in controlling some important soil and seed borne diseases of crop plants. The most important advantage of this is: very much compatible with others, have no adverse effects on others and play synergistic associations with others (Fig. 5).

With the development of fungal pathogen resistance to fungicides, adverse effects of fungicides on natural enemies and public awareness of environmental conservation there has been a renewed interest in the development of crop varieties with resistance to pathogens/pests. Mostly, fungicides produce undesirable effects on non-targeting organisms, so the use of microorganisms that antagonize plant pathogenic fungi is risk free (Benitez *et al.*, 2004). Moreover, the combination of fungicide tolerant biological control agents with reduced levels of fungicide integrated control strategies would promote the degree of disease suppression similar to that achieved with full dosage of fungicides (Monte, 2001). There are reports where the biocontrol agents, which can tolerate fungicides up to a certain level, were mixed with fungicides and resulted in eradication of diseases (De Cal *et al.,* 1994). Integrated disease management incorporating cultural and biological control methods with reduced chemical inputs seems to be a promising approach (Latore *et al.*, 1997). The result of the present screening would help in the selection of biocontrol agents, which can be used, with reduced dose of selected fungicides for the control of soil borne plant pathogenic fungi.

The novelty of this study lies in exploiting a novel strain of *Trichoderma viz., T. aureoviridae* S12 in a fungal consortium along with bacterial consortium. Plant roots support the growth and activities of an array of microorganisms that may impart profound effects on growth and health of plants.

Conclusion

Numerous species of soil fungi and bacteria flourish in the rhizosphere of plants and activate or stimulate plant growth by a plethora of mechanisms. The difficulty of controlling the pathogen lies in the long surviving ability of sclerotia, its broad host range and lack of resistant jute (*Corchorus olitorius* L.). *Trichoderma* spp. is endophytic plant opportunistic symbionts, widely used as biocontrol agents by producing antifungal diffusible and volatile metabolites with antibiotic activity for plant diseases, relatively easy to isolate and ranks top in order of importance besides its positive influence on growth promotion and increased tolerance to various abiotic stresses. Integration of promising plant growth regulator, a bio-fertilizer and bio-control agent is useful for exploitation to achieve higher yield. Inclusion of all such components in the integrated disease management (IDM) is ultimate practical approach for cost effective sustainable agriculture. In the present study, suitable plant growth regulator and chemical fungicide were incorporated to accomplish an acceptable strategy for appropriate IDM system.

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