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In-vivo Evaluation of Agro-Waste based Formulations of Yellow Pigment Producing Actinobacteria against Mulberry Root Rot Pathogens

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Abstract

Mulberry is a multipurpose deciduous tree mainly cultivated for silk cocoon production. The continuous cultivation focusing on high yield made the crop prone to various diseases, especially root rot. To evade the detrimental effects of agrochemicals on sensitive silkworms and environment, the importance was given to bio-control approach. In the present study, four bio-formulations of the actinobacterial isolate NM5 (*Streptomyces parvulus*) were prepared using three carriers: talc, rice husk ash and spent silkworm pupal powder. From the *in-vivo* study against combined inoculation of root rot pathogens, the lowest incidence of disease symptoms including wilting (25.45%) and rotting (20.91%) was observed in APNM5 (rice husk ash: silkworm pupal powder- 1:1 ratio) treated plants which scored as mild to moderate infection. Untreated control was stunted with chlorotic leaves that defoliated prematurely with severe infection (76.25% wilting and 82.80% rotting).

Moreover, in all NM5 treated saplings, as a result of defense action rotten root portions were stimulated to develop new healthy rigid roots. Biometric observations showed formulations had positive effect on plant growth parameters even in the presence of pathogens including higher leaf numbers (27.5), enhanced leaf area (67.96 cm^2) and yield (6 g $plant^{-1}$), shoot length (44.56 cm) and weight 25.50 g $plant^{-1}$), root length (33.67 cm), root weight (2.17 g $plant^{-1}$), root: shoot ratio (0.08) than uninoculated saplings. Therefore, both the performance of potential isolate and effective utilization of agro-waste were enhanced by the nutrient based APNM5 bioformulation in an eco-friendly way.

Keywords: Actinobacteria bioformulation, Agro-waste utilization, Complex root rot pathogens, Mulberry

Introduction

Sericulture is the economic destiny of rural community, which includes the mulberry cultivation and silkworm rearing for the elegant silk production. Plant diseases are one of the major concerns to crop cultivation, especially soil-borne pathogens in perennial crops resulting heavy losses. In India, root rot disease of mulberry recorded plant mortality of 30%, average leaf yield loss of 31.5% (maximum 70%) and reduction in cocoon production by 756 kg ha^{-1} garden (Philip *et al*., 1995; Chowdary and Govindaiah, 2009; Sutthisa *et al.*, 2010).

Root rot disease was endorsed as key disease in mulberry growing areas due to its extensive geographic occurrence and high incidence in severely contaminated soils (Ghosh

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et al., 2017), which also made sericulture farmers to face continuous crop losses. In other leading silk producing countries like China, root rot pathogens resulted in huge loss ($3 million) within a year (Xie *et al*., 2014). The application of agrochemicals tied with its detrimental effects on non-target organisms as many fungicides used in mulberry protection proved toxic to silkworms and environmental pollution (On *et al*., 2015). Thus, there is a prerequisite for an effective sustainable management strategy against the root rot pathogens of mulberry.

Actinobacteria is a filamentous bacterium with the ability to synthesize many bio-active compounds and widely used in management of plant diseases. In addition, it not only affects multiple pathogens at a time and also function as elicitors of defense related proteins in plants (El-Tarabily and Sivasithamparam, 2006; Singh *et al*., 2018; Ruangwong *et al*., 2022).

Similarly, in the preliminary studies, bright yellow pigment producing actinobacteria with significant antifungal activity against root rot pathogens was identified from mulberry rhizosphere. However, the establishment and anti-fungal activity of the potential bio-control agent are manipulated by the environmental and soil conditions. Hence appropriate formulation of the antagonist and biologically active substances are indispensable for successful management of crop diseases.

Based on earlier reports, an attempt was made to mass multiply potential actinobacteria using agro-wastes and their efficiency was evaluated *in-vivo* conditions against mulberry root rot pathogens.

Materials and Methods

Antagonist and Pathogens

Based on the results of preliminary studies, potential anti-fungal actinobacterial isolate NM5 identified from mulberry rhizosphere was used for bio-efficacy evaluation. The pure culture of the isolate NM5 was maintained using starch casein agar (SCA) at 30±2 °C.

The fungal pathogens associated with mulberry root rot disease were obtained during the survey (2019-2021). Based on their virulence, representative isolate of each pathogen *viz., Fusarium solani* (KV1), *Lasiodiplodia theobromae* (DP1), *Athelia rolfsii* (SR1) and *Macrophomina phaseolina* (MPCK2) were used in the study. The pure culture of the isolates was maintained using Potato dextrose agar (PDA) at 28±2 °C.

Bio-formulations of Actinobacteria

Inocula of actinobacterial isolate (NM5) were prepared by adding 10 ml pre-cultured broth to 250 ml of sterile starch casein broth and incubated for 10-15 days at 30±2 °C. Four formulations were prepared using three carriers as described with slight modifications (Adhilakshmi *et al*., 2013; Zamoum *et al.,* 2017). Apart from the inert carrier material talc, sericultural and agricultural residues were used as nutritive carriers (Patil *et al*., 2013; Sarin and Riddech, 2018). The selected carriers and formulations were talc (T), rice husk ash (A), talc: silkworm pupal powder (TP - 1:1 ratio) and rice husk ash: silkworm pupal powder (AP - 1:1 ratio).

Silkworm pupae obtained from reeling units were oven dried at 80 °C till reaching constant weight and ground (Karthikeyan and Sivakumar, 2007). Rice husk ash (A) was obtained from rice mills were used for formulation. To the sterilized carrier materials (one kg), actinobacteria culture broth (400 ml) was mixed along with 5 g carboxy-methyl cellulose (CMC) as filler and sticking agent. The moisture content less than 20% was packed and stored at 28±2 °C. The four formulations of NM5 isolate were screened for the *in-vivo* biocontrol efficacy against mulberry root rot pathogens.

In-vivo Evaluation of Actinobacteria Bio-formulations

Two months old mulberry saplings were transferred to the pots filled with 3 kg of potting mix. The potting mix (FYM: sand: soil - 1:1:1) was sterilized by autoclaving twice at 121 °C for 20 min on consecutive days. Actinobacteria formulations were applied (25 g plant^{-1}) at the time of transplanting to bio-harden the mulberry saplings.

The auxenic culture of all pathogens *viz., M. phaseolina* (MPCK2), *F. solani* (KV1), *L. theobromae* (DP1) and *A. rolfsii* (SR1) were mass multiplied in overnight soaked sorghum grains as mentioned (Pinto *et al.*, 2018). After 30 days of transplanting, mixture of pathogens inocula in sorghum grains was prepared in equal ratio (1:1:1:1). For inoculation of pathogens mixture, soil near roots was carefully removed and pathogens mixture was applied at the rate of 3% (w/w) to all bio-hardened saplings (Pinto *et al*., 2018; Porto *et al*., 2020). The details of treatments were listed below (Table 1).

Table 1: Treatment details

Sl. No.	Formulation details	Code
1	Talc formulation of NM5 + Pathogens mixture	TNM5
2	Talc + pupal powder formulation of NM5 + Pathogens mixture	TPNM5
3	Rice husk ash formulation of NM5 + Pathogens mixture	ANM5
4	Rice husk ash + pupal powder formulation of NM5 + Pathogens mixture	APNM5
5	Uninoculated control (Positive)	PC
6	Untreated control (Pathogen inoculated/ negative)	NC

Three replications per treatment along with positive (uninoculated) and negative (pathogens alone) controls were maintained in completely randomized block design (CRBD). Positive control was loaded with uninoculated sterile sorghum grains; whereas, negative control with pathogens mixture as that of treatments. Irrigation twice a week was followed by maintaining soil moisture content (below 40%) using soil moisture sensor (ML3 Theta probe, Delta-T devices, UK).

Observations like percent of sprouting (at 10 days after planting DAP), survival, wilting, rotting and biometric/

growth parameters of the mulberry saplings were recorded at 90 days after inoculation (DAI).

Results and Discussion

A broad spectrum actinobacteria NM5 had been isolated from healthy mulberry rhizosphere which found to inhibit the fungal pathogens associated with mulberry root rot disease. It exhibited significant antifungal activity of more than 50% reduction over control against virulent root rot pathogens in dual culture.

Morphologically the isolate NM5 was grey in colour with pale white substrate mycelia strongly rooted in agar media (Figure 1), fast growing and produced diffusible bright yellow pigment in 5 DAI. Based on their gene sequence, this potent isolate was molecularly confirmed as, *Streptomyces parvulus* and also deposited in NCBI GenBank (OL657043).

Similar to our results, Silva-Lacerda *et al.* (2016), had isolated potential anti-fungal actinobacteria from the rhizosphere of Catingueira and identified as *S. parvulus* C1.129. Anti-bacterial efficacy of the organism (*S. parvulus* RSPSN2) obtained from marine samples was recorded by the production of polypeptide actinomycin D (Shetty *et al.,* 2014). Further, Soundari *et al.* (2017) mentioned the presence of bioactive fractions in the yellow pigment of *S. parvulus* C5-5Y.

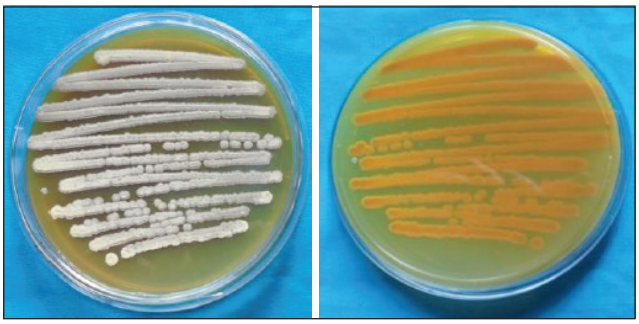

Figure 1: Adaxial and abaxial side of NM5 on SCA with diffusible yellow pigment production

Bio-formulations of Actinobacteria Isolate (NM5)

Fully grown 400 ml culture broth of NM5 was evenly mixed with one kg autoclaved carrier materials *viz.,* talc (T), rice husk ash (A), talc: silkworm pupal powder (TP - 1:1 ratio) and rice husk ash: silkworm pupal powder (AP - 1:1 ratio). These four formulations were air dried, labeled, packed (< 20% moisture content) and stored at room temperature (28±2 °C) (Figure 2a).

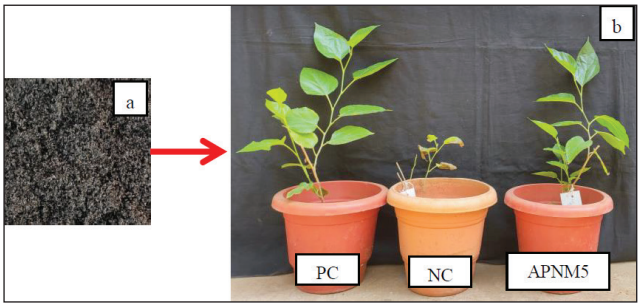

Figure 2: (a) Rice husk ash: Silkworm pupal powder (APNM5) formulation; (b) In-vivo evaluation of actinobacteria bio-formulation against mulberry root rot pathogens

In this study, potential isolate NM5 was mass produced using agro-waste as carriers and evaluated against four root rot pathogens of mulberry with talc based formulation as standard. Based on earlier reports, an attempt was made to utilize sericulture waste - silkworm pupal powder for enhancing storage and anti-fungal efficacy of actinobacteria. Pupal powder was rich in protein (47% dry weight) and lipids (45% dry weight) which acted as carbon and nitrogen sources, thus enhanced the shelf life of *Bacillus thuringiensis* (Karthikeyan and Sivakumar, 2007; Patil *et al*., 2013). Further, Sarin and Riddech (2018) mentioned rice husk ash out performed than the other three types of carriers (rice straw, sugarcane leaves, and coconut coir) in terms of growth and survival of rhizobacteria. Sabaratnam and Traquair (2002) also found talc as a better carrier for *Streptomyces* sp. Di-944 formulation for the management of tomato diseases. Further, bio-conversion of agro-processing residues such as rice husk ash, coffee husk, bagasse for the mass production of fungal BCAs was mentioned as economically feasible processes (Das and Abdulhameed, 2020).

In vivo Evaluation of Actinobacteria Bio-formulations against Mulberry Root Rot Pathogens

The best performing isolate NM5 was formulated in four different carriers and was tested against root rot disease in mulberry saplings under artificially inoculated condition.

The results showed that all the formulations (TNM5, TPNM5, ANM5 and APNM5) reduced disease incidence significantly (P≤0.05) compared with untreated/ negative control (Table 2, Figure 2b). All the bio formulations treated plants showed 100% survivability at 90 DAI; however, mortality of 66.67% was recorded in negative control. Significant reduction of wilting symptoms to 25.45% in APNM5 treated plants while untreated control plants had showed maximum of 76.25% wilted foliage. All other NM5 formulation treated plants showed more than 50% reduction in wilting symptoms over control.

In all NM5 treated plants, rotten root portions were stimulated to develop new healthy rigid roots (Figure 3c) marked by red arrow. Lowest incidence of root rot was observed in APNM5 (20.91%) treated plants where the negative control showed maximum rotting of 82.8% at 90 DAI. This was 74.7% reduction over untreated control. Based on the observations, disease incidence in treated and untreated plants was scored as mild and very severe, respectively. The remining treatments reduced rotting from 71 to 59% over untreated control and showed mild to moderate infections in plants.

Combined inoculation of pathogens MPCK2, KV1, DP1 and SR1 in negative control plants resulted in 82.8% disease incidence, which was graded as very severe. Untreated control was stunted with chlorotic leaves that defoliated prematurely and these symptoms matched with actual root rot symptoms observed under field. Up on treatment with four different formulations, the disease incidence significantly reduced and maximum reduction of rotting and wilting over untreated control was seen in APNM5 as

74.75 and 66.61%, respectively. Similarly soil application of powder formulations of all three strains of *Streptomyces* sp. to groundnut substantially reduced the incidence of stem rot by 72.2% (Adhilakshmi *et al*., 2013).

Bio-formulation of *Streptomyces rochei* PTL2 reduced tomato damping off from 89.3% to 14.1% (Zamoum *et al.*, 2017). Talc formulation of *Streptomyces* sp. RP1A-12 outperformed crude extract formulation by reducing groundnut stem rot disease severity (DS) to 2.2% with yield of 17.5 g pot^{-1}; whereas, pathogen inoculated plants recorded 5% DS and zero yield (Jacob *et al.*, 2016). Boukaew *et al.* (2011) used a bioformulation of *S. philanthi* RL-1-178 to control root and stem rot of chili pepper.

Table 2: In vivo evaluation of actinobacteria bioformulations against mulberry root rots pathogens

Sl. No.	Treatment*	Sprouting (%) (10 DAP)	90 DAI			
			Wilting (%)	Rotting (%)	Survival (%)	Disease score
1	TNM5	100.00	36.76^{d}	32.09^{c}	100.00^{a}	Moderate
2	TPNM5	100.00	28.57^{c}	23.71^{b}	100.00^{a}	Mild- moderate
3	ANM5	100.00	37.50^{d}	34.75^{c}	100.00^{a}	Moderate
4	APNM5	100.00	25.45^{b}	20.91^{b}	100.00^{a}	Mild- moderate
5	PC	100.00	0.00^{a}	0.00^{a}	100.00^{a}	Healthy
6	NC	100.00	76.25^{e}	82.80^{d}	33.33^{b}	Very severe
S.Ed.	NS	0.556	1.739	2.666	-	
CD (P≤0.05)	NS	1.168	3.654	5.810	-	

* TNM5 - Talc formulation; TPNM5 - Talc + pupal powder formulation; ANM5 - Rice husk ash formulation; APNM5 - Rice husk ash + pupal powder formulation; PC - Positive control; NC - Negative control; DAP - Days after planting; DAI - Days after inoculation

Effect of Actinobacteria Bioformulations on Biometric Parameters of Mulberry Saplings

Biometric observations showed formulations had positive effect on plant growth parameters even in the presence of root rot pathogens (Table 3). Leaf numbers were non-significantly higher in APNM5 (27.50) and TPNM5 (21.00) treated plants than in uninoculated plants (17.00). Except the treatment ANM5 all other treatments had enhanced leaf area than untreated control that ranged from 67.96 to 76.46 cm^2; however, lower than positive control (90.40 cm^2). Leaf yield was noticed high in uninoculated plants (7.33 g plant^{-1}) followed by APNM5 (6 g plant^{-1}) treated plants.

Table 3: Effect of actinobacteria bioformulations on biometric parameters of mulberry saplings

Sl. No.	Treatment**	90 DAI							
		L (nos')*	LY (g)*	LA (cm2)*	SL (cm)*	SW (g)*	RL (cm)*	RW (g)*	R:S ratio*
1	TNM5	17.00^{bc}	4.50^{d}	71.51^{b}	39.23^{bc}	22.67^{c}	24.33^{e}	1.00^{d}	0.068^{c}
2	TPNM5	21.00^{ab}	6.50^{b}	76.46^{b}	40.56^{bc}	24.83^{b}	25.00^{d}	1.67^{b}	0.067^{c}
3	ANM5	16.00^{bc}	3.00e	31.71^{c}	35.46^{bc}	19.67^{e}	28.00b	1.00^{d}	0.060^{d}
4	APNM5	27.50^{a}	6.00^{c}	67.96^{b}	44.56^{ab}	25.50^{a}	33.67^{a}	2.17^{a}	0.08^{a}
5	PC	17.00^{bc}	7.33^{a}	90.40^{a}	52.00^{a}	20.83^{d}	27.25^{c}	1.50^{c}	0.072^{b}
6	NC	10.66^{c}	2.33^{f}	37.12^{c}	32.33^{c}	16.50^{f}	11.33^{f}	0.50^{e}	0.030^{e}
S.Ed.		3.345	0.066	6.098	4.603	0.263	0.313	0.053	0.001
CD (P≤0.05)		7.028	0.139	12.812	9.672	0.552	0.658	0.110	0.002

** TNM5 - Talc formulation; TPNM5 - Talc + pupal powder formulation; ANM5 - Rice husk ash formulation; APNM5 - Rice husk ash + pupal powder formulation; PC - Positive control; NC - Negative control; DAI - Days after inoculation.
* L - Number of leaves; LY - Leaf yield; LA - Leaf area; SL - Shoot length; SW - Shoot weight; RL - Root length; RW - Root weight; R:S ratio - Root shoot ratio

APNM5 bioformulation treated plants had shoot length (44.56 cm) on par with uninoculated control plants; whereas, significantly increased shoot weight was noticed in APNM5 (25.50 g plant^{-1}) over positive control.

Root parameters like root length (33.67 cm), root weight (2.17 g plant^{-1}) and root shoot ratio (0.08) were higher in APNM5 treated plants than positive control plants (27.25 cm, 1.50 g plant^{-1}, 0.072 respectively). The enhanced root architecture of the saplings (length and width) could be visualized in all NM5 treated plants than healthy plants (Figure 3c) marked by red arrow. The roots of saplings were remained healthy and completely disintegrated in positive

and negative control, respectively (Figure 3a and 3c). The results clearly showed the synergistic effect of rice husk ash and pupa (APNM5) formulation performed well over other treatments.

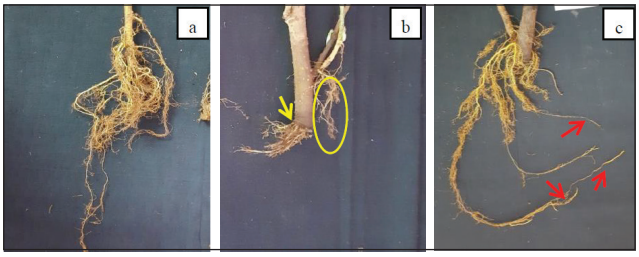

Figure 3: Root architecture of mulberry saplings: (a) PC, (b) NC, (c) APNM5 -regenerated root portion indicated by red arrow

Mulberry leaf is an important economic component in sericulture since the quality and quantity of leaf generated per unit area influences cocoon harvest. Mulberry charcoal rot resulted in loss of leaf yield (35%), drop in leaf size, deterioration of leaf quality and plant death (Chowdary and Govindaiah, 2009). Root rot disease of mulberry had a negative impact on sericulture profitability (Pinto *et al.*, 2018). Guha *et al.* (2010) mentioned that leaf area was the major economic unit of the mulberry crop. In our study, NM5 treated saplings showed good leaf area even after pathogen inoculation. Moreover, shoot weight was increased over positive control in these treatments. However, in root parameters APNM5 was out performed well than healthy control. Similar results were obtained by Zaid *et al*. (2021) as the talc-based formulation increased the weight of peanut shoot (41.56 g) and root (4.59 g) more dramatically than other treatments and untreated control (*A. rolfsii* alone). Zamoum *et al.* (2017) highlighted the positive impact on tomato seedlings in the presence of damping off pathogen *R. solani* as increase in root length, shoot length, dry weight due to the treatment of *S. rochei* PTL2 bio-formulation.

Between the treatments, there were significant changes in root: shoot ratio. Among the formulations, APNM5 expressed higher root shoot ratio R:S (0.08) when compared to healthy plants. Guha *et al.* (2010) also mentioned increased R:S under drought stress in all mulberry varieties as compared to control, especially in V1. Increased R:S, might be the indirect indication that saplings were under stress (inoculated with pathogens). Liu *et al*. (2019) also mentioned the increase in R:S ratio of mulberry from 0.12 to 0.26 under stressed conditions.

While observing root architecture of treated and untreated mulberry saplings, the rotten root portion were regenerated into healthy rigid root portion. In root diseases, it was common for soil-borne pathogens to attack feeder roots followed by tap roots (Govindaiah *et al*., 2005). This clearly depicted the defense actions of NM5 based bio-formulations on mulberry root rot pathogens. They helped the plants by enhancing the regeneration ability especially in the root portions, the main zone of infection by pathogens.

Conclusion

Due to intensive cropping, the same piece of land is used for cultivation which deteriorates soil health extremely and left the farmers with no choice expect to depend on agro-chemicals. However, continuous use of excessive agro-chemicals led to resistance development in primary and secondary pathogens as well. Hence it is foremost important to protect both soil and plants by biological, eco-friendly means.

In this study, the antifungal efficacy of potential antifungal isolate NM5 was improvised using nutrient based carriers, however, reduction in wilting and rotting % was prominent over negative control. This actinobacterial isolate was identified as *S. parvulus* obtained from mulberry rhizosphere, once again proved rhizosphere as an excellent source of novel, anti-microbial microbes. Apart from reducing disease incidence in mulberry plants, they also had positive effect on plant growth parameters and visualized the regeneration of infected root portions in NM5 treated plants.

Across the world tonnes of agro-waste were generated from each square of the farm land and were improperly disposed or recycled. Therefore, both the performance of potential isolate and effective utilization of agro-waste could be achieved by the nutrient based *S. parvulus* (NM5) bioformulation in an eco-friendly way.

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