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ANTIFUNGAL EFFECT OF MYCO-SYNTHESIZED SILVER NANOPARTICLES AGAINST ANTHRACNOSE OF TROPICAL FRUIT CROPS

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ABSTRACT

Anthracnose, caused by *Colletotrichum* spp. leads to significant pre- and post-harvest losses in tropical fruit crops by reducing the yield, fruit quality, consumer preferability, and market value. Prevalence of one or more *Colletotrichum* species in fruit orchards pose a difficulty in their management. Hence, a novel management strategy was explored by testing the antifungal effect of myco-synthesized silver nanoparticles. Three species viz., *C. musae*, *C. asianum* and *C. gloeosporioides* were identified from anthracnose samples of banana, mango and pomegranate respectively. *Trichoderma asperellum* mediated myco-synthesized silver nanoparticles (Tas-AgNPs) of 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml concentrations were evaluated. Spore inoculation assay was conducted by pathogen inoculation with 1×10^6 conidia/ml followed by simultaneous application of Tas-AgNPs on detached leaf/fruit of host. At 100 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 37.33 for banana, 43 for mango and 34.33 for pomegranate. At 150 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 24.66 for banana, 27.33 for mango and 28.33 for pomegranate. At 200 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 14.33 for banana, 18 for mango and 18.33 for pomegranate. At 250 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 10.33 for banana, 10.66 for mango and 12.33 for pomegranate. At 300 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 6.33 for banana, 7.66 for mango and 6.33 for pomegranate. As the concentrations of Tas-AgNPs was increased, the mean lesion count was decreased indicating that higher Tas-AgNPs concentrations significantly inhibited anthracnose. No signs of phytotoxic reactions of 300 µg/ml Tas-AgNPs were observed in fruit surface of control treatments (T8) of banana, mango and pomegranate. Therefore, the *T. asperellum* mediated myco-synthesized silver nanoparticles can used for effective management of anthracnose of tropical fruit crops.

***Keywords*:** Anthracnose, Antifungal, Mycosynthesis, Silver nanoparticles, Tropical fruits

Introduction

India is a leading producer of tropical fruit crops in the world. Tropical fruit crops like banana, guava, mango, papaya, pomegranate is of high commercial value. The production of tropical fruit crops was affected by several biotic and abiotic factors. Among the biotic factors, the fruit rot pathogens especially *Colletotrichum* spp. cause anthracnose disease and leads to substantial economic losses during both pre- and post-harvest stages. *Colletotrichum* spp. are recognized as causal agent for the post-harvest fruit rots of many hosts. Huge yield losses were caused by the damage caused in post-harvest infection by *Colletotrichum* spp., in fruit crops. This may be attributed to the brief initial biotrophic phase, where the pathogen inoculum already enters the host plants and prolonged necrotrophic phase, where the symptoms and disease development cause damage and significant economic losses (O'Connell *et al*., 2012). Plant pathologists ranked *Colletotrichum* spp. as the eight most important plant pathogen in the world (Dean *et al*., 2012). The shelf life of tropical fruit crops

was relatively low in comparison to food crops. Anthracnose was observed on different plant parts of tropical fruit crops such as roots, leaves and fruits of banana (Nazriya *et al*., 2007), unripe green fruits of mango (Kumari *et al*. (2025), leaves, stems, pedicels, floral buds, flowers and fruits of pomegranate (Xavier *et al*., 2019). The infection of anthracnose progressed into the internal tissues of the fruits causing decaying of the fruits (Masyahit *et al*., 2009; Manjunatha *et al*., 2022). Asymptomatic survival in fruit crops was also observed by *Colletotrichum* spp. (Shetty *et al*., 2016; Guarnaccia *et al*., 2017). Management of *Colletotrichum* spp. was difficult because they exhibit different life styles in fruit crops such as endophytic, necrotrophic, hemi-biotrophic and also with quiescent or latent infection phase (De Silva *et al*., 2017). Broad host range of *Colletotrichum* spp. and their ability to cross infect other fruit crops further complicate their management (Lakshmi *et al*., 2011; Teja *et al*., 2022). This diversified existence of *Colletotrichum* spp. has significant implications for plant biosecurity. The significant challenges posed by *Colletotrichum* spp. requires the development of sustainable and innovative management strategies. Nanotechnology offers a promising and effective plant disease management. Nanoform of application of metal particles gives effective control of plant pathogens. Nanoparticles synthesized using green approaches using *Trichoderma* spp. metabolites as reducing agent provides and ecofriendly synthesis. Myco-synthesized nanoparticles have metal ions capped by the fungal extracts rich in metabolites. Such metal-based nanoparticles offer dual antagonism effects using properties of both metal ions and metabolites. Nanoform of silver metal is well known for its antimicrobial nature (properties) and used against phytopathogens. Antifungal efficacy of myco-synthesized silver nanoparticles against *Colletotrichum* spp. was less explored and needs to be exploited for novel disease management. Hence, the present study was conducted to evaluate the antifungal efficacy of myco-synthesized silver nanoparticles against anthracnose of tropical fruit crops.

Material and Methods

The experiments were conducted during the *Rabi* season, 2024 at the laboratory of Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

Colletotrichum species and their host crops

Three species viz., *C. musae, C. asianum, C. gloeosporioides* were identified from anthracnose symptoms of banana, mango and pomegranate respectively. These pathogens were maintained in potato dextrose agar (PDA) and incubated at 25 ± 1 °C in 12 hours of alternating light and dark cycles. These three species were utilized for the antifungal efficacy studies of Tas-AgNPs.

Preparation of spore suspension

Three species were grown in pure culture in PDA for period of 10 days until mature conidial masses were visible in the culture plates. Then, the conidial masses were collected by adding 5 ml of sterile distilled water to the culture petri dishes and swirled to cover the movement of water through entire petri dish. The conidial masses were lifted and moved along with the movement of the water. These harvested conidial masses were poured out into a test tube. Then, the conidia count was made using haemocytometer. The conidial count was adjusted to 1×10^6 conidia/ml by dilution.

Spore inoculation assay

The healthy, susceptible leaves of mango and matured but unripe fruits of banana and pomegranate were selected for the assay. They were washed under running tap water followed by surface sterilization with 1% sodium hypochlorite (NaOCl) for 3 minutes and rinsing twice with sterile distilled water. They were dried on blotting papers. These leaves and fruits were placed in plastic boxes with blotting paper and moist cotton balls to provide relative humidity for disease development. The spore suspension of 1×10^6 conidia/ml of respective test pathogen was pipetted out and spread across the inoculating surface of the leaf/fruit using a L shaped glass spreader for smooth and uniform spreading of the spore suspension. Direct inoculation was done without any wounding to leaves/fruits to prevent confusion of bruises with the anthracnose lesions.

Preparation of Tas-AgNPs

The *T. asperellum* mediated myco-synthesized silver nanoparticles were utilized for the preparation of various concentrations of Tas-AgNPs. The synthesized Tas-AgNPs powder was dispersed in distilled water. 10 mg, 15 mg, 20 mg, 25 mg, 30 mg of Tas-AgNPs powder was weighed on analytical weighing balance and dispersed in 100 ml each resulting in final concentrations of 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml. A probe based ultra-sonication of 15 min for 1 cycle was done for all the prepared concentrations of Tas-AgNPs for uniform dispersion and breaking the aggregates of Tas-AgNPs.

Antifungal efficacy of Tas-AgNPs

Application of concentrations of 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml of well dispersed Tas-AgNPs was done on the surface of the leaves and fruits immediately after the spore inoculation and uniformly spread using L shaped glass spreader. Three leaves/fruits per treatment were tested. Three controls were used. Control (T6) was inoculated with only distilled water on the leaf/fruit, Control-2 (T7) was inoculated with only 1×10^6 conidia/ml conidial suspension and Control-3 (T8) was application of only 300 µg/ml of Tas-AgNPs. The treatment details were presented in Table 1. For all treatments, the lesion count of anthracnose was done on 7th day post inoculation for mango and pomegranate and on 3rd day post inoculation for banana due to early ripening nature of the fruit.

Table 1: Treatment details of antifungal efficacy of Tas-AgNPs

Treatment Name	Treatments
T1	1×10^6 conidia/ml + 100 µg/ml of Tas-AgNPs
T2	1×10^6 conidia/ml + 150 µg/ml of Tas-AgNPs
T3	1×10^6 conidia/ml + 200 µg/ml of Tas-AgNPs
T4	1×10^6 conidia/ml + 250 µg/ml of Tas-AgNPs
T5	1×10^6 conidia/ml + 300 µg/ml of Tas-AgNPs
T6 (Control-1)	Sterile distilled water
T7 (Control-2)	1×10^6 conidia/ml
T8 (Control-3)	300 µg/ml of Tas-AgNPs

Statistical analysis

The statistical analysis was performed for two factors i.e., factor-1 was Tas-AgNPs treatments and factor-2 was Fruit crops. The data of factors and their interactions were analysed with completely randomized design using two-way analysis of variance (2-way ANOVA) and the significance of treatment means was compared at 1% level of significance ($p < 0.01$). The statistical analysis was performed in Online Statistical Analysis tool (OPSTAT- http://opstat.somee.com/opstat/) (Sheoran *et al*., 1998).

Results and Discussion

The measure of antifungal efficacy was recorded through the anthracnose lesion count on the fruit surface, while lesion count was in turn a measure of conidial germination on the fruit surface. Figure-1 represents antifungal efficacy of Tas-AgNPs treatments against *C. musae* on banana. Figure-2 represents antifungal efficacy of Tas-AgNPs treatments against *C. asianum* on mango. Figure-3 represents antifungal efficacy of Tas-AgNPs treatments against *C. gloeosporioides* on pomegranate. The results data was presented in Table 2. At 100 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 37.33 for banana, 43 for mango and 34.33 for pomegranate. The maximum inhibition of conidial germination by 100 µg/ml Tas-AgNPs was against *C. gloeosporioides* on pomegranate followed by *C. musae* on banana. The least inhibition of 100 µg/ml of Tas-AgNPs was against *C. asianum* on mango. At 150 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 24.66 for banana, 27.33 for mango and 28.33 for pomegranate. The maximum inhibition of conidial germination by 150 µg/ml Tas-AgNPs was against *C. musae* on banana followed by *C. asianum* on mango. The least inhibition of 150 µg/ml of Tas-AgNPs was against *C. gloeosporioides* on pomegranate.

At 200 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 14.33 for banana, 18 for mango and 18.33 for pomegranate. The maximum inhibition of conidial germination by 200 µg/ml Tas-AgNPs was against *C. musae* on banana followed by *C. asianum* on mango. The least inhibition of 200 µg/ml of Tas-AgNPs was against *C. gloeosporioides* on pomegranate. At 250 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 10.33 for banana, 10.66 for mango and 12.33 for pomegranate. The maximum inhibition of conidial germination by 250 µg/ml Tas-AgNPs was against *C. musae* on banana followed by *C. asianum* on mango. The least inhibition of 250 µg/ml of Tas-AgNPs was against *C. gloeosporioides* on pomegranate. At 300 µg/ml of Tas-AgNPs, the mean anthracnose lesion counts were 6.33 for banana, 7.66 for mango and 6.33 for pomegranate. The maximum inhibition of conidial germination by 300 µg/ml Tas-AgNPs was against *C. musae* on banana and *C. gloeosporioides* on pomegranate followed by *C. asianum* on mango.

The observations were similar for control treatment of T1, where no symptoms of anthracnose lesions on banana, mango and pomegranate were recorded because of only distilled water inoculation. T1 was performed to ensure that the distilled water used doesn't cause any specific reactions on the fruit surface and also to understand no natural anthracnose symptoms were developed. T2 displayed numerous anthracnose lesions on banana, mango and pomegranate indicating high germination of conidia from spore suspension. T2 indicates the vigorous nature of conidia of test pathogens when inoculated alone without any Tas-AgNPs and also acts as a reference for anthracnose lesions to compare with the treatments and understand the ability of the Tas-AgNPs to inhibit the conidial germination and lesion

formation. T3 displayed no symptoms of phytotoxicity reactions on the fruit surfaces of banana, mango and pomegranate. T3 control was set up for 300 µg/ml of Tas-AgNPs in order to test that higher concentrations of Tas-AgNPs may show some phytotoxic reactions and symptoms. No such phytotoxic symptoms were visible on leaves/fruits of T3 control.

Therefore, as the concentration of the Tas-AgNPs was increased, the lesion count was decreased which indicates the effective inhibition of conidial germination and anthracnose lesion development. The line graph of Figure-4 clearly depicted the decreasing lesion count of against increasing concentrations of Tas-AgNPs in all three fruit crops. The results also displayed that Tas-AgNPs have uniform antifungal efficacy per each concentration among all the three *Colletotrichum* spp. This uniformity in antifungal efficacy results was estimated due to their common evolutionary origin in phylogenetic clade of *Colletotrichum gloeosporioides* species complex. This increased antifungal efficacy with higher concentrations of Tas-AgNPs may be attributed to availability of a greater number of stable silver nanoparticles. As the concentration of Tas-AgNPs increases which means the quantity/number of the Tas-AgNPs dispersed was also increased. Tas-AgNPs inhibit or kill the germinating conidia of the applied spore suspension.

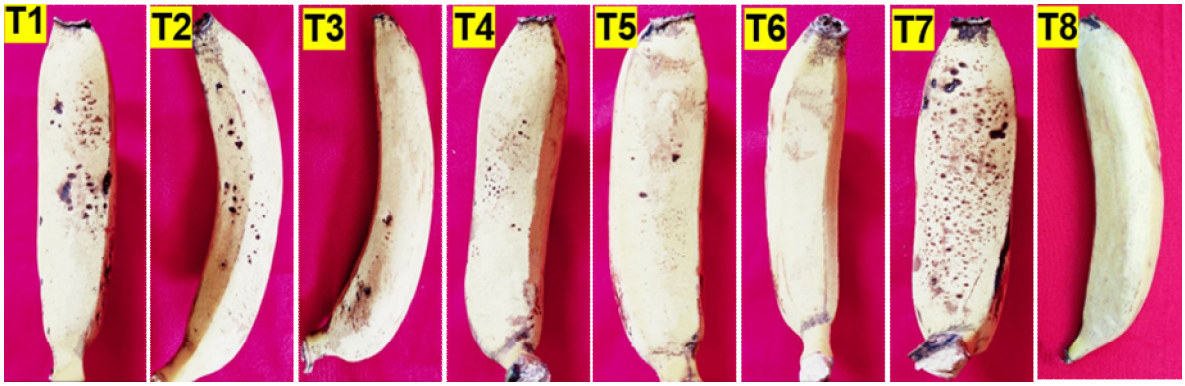

Fig. 1: Antifungal efficacy studies of Tas-AgNPs against *C. musae* on banana

Fig. 2: Antifungal efficacy studies of Tas-AgNPs against *C. asianum* on mango

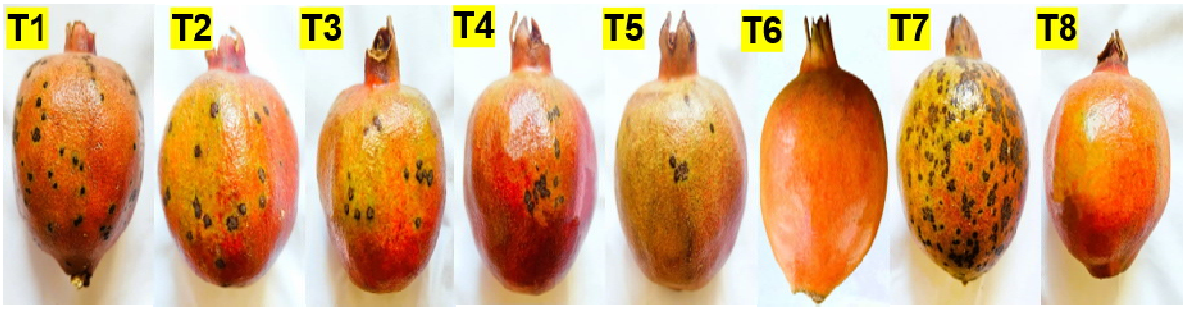

Fig. 3: Antifungal efficacy studies of Tas-AgNPs against *C. gloeosporioides* on pomegranate

Table 2: Antifungal efficacy of Tas-AgNPs against *C. asianum*, *C. musae* and *C. gloeosporioides* using spore inoculation assay

Tas-AgNPs Treatments (Factor-1)	Fruit crops (Factor-2)			Mean of factor-1 (Tas-AgNPs)
	C. musae **(Banana)**	***C. asianum*** **(Mango)**	***C. gloeosporioides*** **(Pomegranate)**	
T1	37.33	43	34.33	38.22
T2	24.66	27.33	24.66	25.55
T3	14.33	18	15.33	15.88
T4	10.33	10.66	11.66	10.88
T5	6.33	7.66	5.00	6.33
Mean of factor-2 (Fruit crops)	18.60	21.33	18.20	-
-	**Tas-AgNPs (F1)**	**Fruit crops (F2)**	**Interaction of Factors (1 ×2)**	-
SE ± m	0.51	0.40	0.89	-
C.D. (1%)	1.49	1.16	2.59	-

In lower concentrations of Tas-AgNPs, the availability of nanoparticles was lesser in quantity which was not sufficient to completely inhibit all the germinating conidia. At higher concentrations, the Tas-AgNPs were available more in quantity which allows maximum inhibition of the germinating conidia. The mechanism underlying the antifungal efficacy of Tas-AgNPs against germinating conidia in spore inoculation assays may be attributed to the cause of damage to the walls of conidia, cause structural damage to the germ tubes and appressoria or may form a biofilm like network on the leaf/fruit surface preventing hyphal penetration.

The antifungal efficacy of the Tas-AgNPs may also be attributed to the nanoscale of silver ions along with metabolites because of the nano-size, the surface area of the silver nanoparticles was large in comparison to the regular bulk silver in $AgNO_3$. Since, the metabolites capped silver ions form nano-size which in turn increased the surface area of the metal thereby enhancing the antimicrobial activity. The nanoscale of the Tas-AgNPs allow them to quickly and easily penetrate the walls of conidia of test pathogen leading to leakage of the contents of the conidia and cause disruption of conidia. Also, Tas-AgNPs may also interact as reactive oxygen species (ROS) which reacts highly with cellular molecules of conidia and scavenge them, ultimately causing mortality of the conidia of test fungi. The surface charge of the silver ions may also cause different interactions and bonds forming with the conidial cellular molecules and thereby disrupting their function. Also, these silver metal ions were capped by the powerful metabolites of *T. asperellum* which also has the antifungal properties. The dual combination of antifungal properties of both silver ions and capping metabolites of *T. asperellum* cause synergistic effect of fungicidal or fungistatic actions. Therefore, Tas-AgNPs can be utilised in effective anthracnose disease management of tropical fruit crops in this study.

Antifungal efficacy of biosynthesized silver nanoparticles against anthracnose was also reported by earlier researchers. Disease incidence was lowest (9.7%) recorded with 50 µg/ml silver nanoparticles on pepper plants before the outbreak of anthracnose caused by *C. gloeosporioides* indicating the potential inhibitory effect of silver nanoparticles (Lamsal *et al*., 2011). Germination of conidia of *C. gloeosporioides* were significantly inhibited by 0.1 µg/ml, 1 µg/ml, and 10 µg/ml by 44%, 70% and 78% respectively. Complete spore germination was inhibited at 100 µg/ml. 0.5% and 1% concentrations of chitosan-AgNP composite were significantly reducing the inoculated spore suspension of 1.5×10^6 conidia/ml of *C. gloeosporioides* by 45.7% and 71.3% respectively on mango fruits (Chowdappa *et al*., 2014). Different concentrations of AgNPs viz., 0.001%, 0.002%, 0.004%, 0.008%, 0.015%, 0.031%, 0.062%, 0.125%, 0.25%, 0.5% and 1% against *C. truncatum* displayed 100% conidial germination inhibition while concentrations below 0.001% recorded conidial growth. As the AgNPs concentration increases, the growth of conidial inhibition of *C. truncatum* also increased. AgNPs also inhibited appressoria formation Inhibitions of conidial germination and appressoria may be accounted due to small size, stability and high surface area of AgNPs, cell structure permeability damage, leakage of cellular contents and melanin content reduction (Gowda and Sriram, 2023). Spraying

of 50 mg/L, AgNPs on common bean leaves reduced 70% conidial germination of the inoculated spore suspension of 4×10^6 conidia/ml and melanisation of appressoria of *C. lindemuthianum* (dos Santos *et al*., 2024).

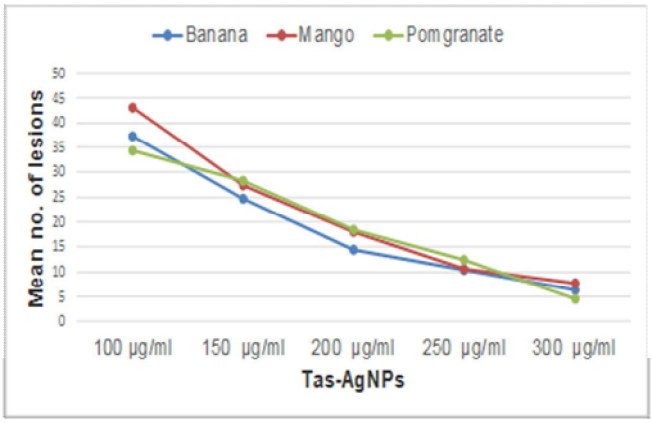

Fig. 4: Antifungal efficacy of Tas-AgNPs in different concentrations against three *Colletotrichum* species on their respective hosts

Conclusion

Anthracnose disease of tropical fruit crops causes significant yield and economic losses to the producers at both pre- and post-harvest stages. Innovative approaches were required for sustainable disease management of anthracnose in tropical fruit crops. *T. asperellum* mediated myco-synthesized silver nanoparticles (Tas-AgNPs) of varying concentrations were tested against three species of *Colletotrichum* viz., *C. asianum, C. musae* and *C. gloeosporioides* infecting banana, mango and pomegranate. Lowest disease reduction was found in 100 µg/ml of Taas-AgNPs while highest disease reduction was found in 300 µg/ml Tas-AgNPs. Increasing disease reduction was recorded with increase in concentrations of Tas-AgNPs. Also, no symptoms of phytotoxicity were observed at maximum concentration of 300 µg/ml Tas-AgNPs used in this study. Therefore, Tas-AgNPs can be used as a novel ecofriendly approach for management of anthracnose of tropical fruit crops.

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