The efficacy of coating anthracnose-infected chili seed with 1-deoxynojirimycin (1-DNJ) extracted from mulberry leaves on seed germination and seedling growth

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Chilli pepper is one of the most important economic crops. However, anthracnose (*Colletotrichum* spp.) is one of the most devastating fungal diseases affecting the quality and yield of chili. There is a need to control this fungal infection at all growth stages, starting from the seed (initial) stage, by using a natural and eco-friendly approach. Laboratory and pot studies were conducted to evaluate the efficacy of coating anthracnose-infected chili seed with 1-Deoxynomyjirimycin (1-DNJ) mulberry leaf extract on seed germination, plant growth, and anthracnose development. The levels of 1-DNJ mulberry leaf extract coating were 1, 2, 3 and 4%. In addition, a positive control with 1% thiram fungicide was applied as well as a negative control with neither 1-DNJ nor thiram application. The results revealed that coating chilli seed infected with anthracnose with the mulberry leaf extract resulted in a significantly improved germination rate of over 80% in treatments 2, 3, and 4% mulberry leaf extract coating. The chilli plant growth parameters, root lengths and shoot heights were significantly greater in the treatments where seeds were coated with 2, 3 and 4% mulberry leaf extract coating compared to both positive and negative controls. A similar result was observed for chilli seedlings shoot fresh weight, where the shoot fresh weight was the highest in the treatment with 2% mulberry leaf extract. These results clearly showed that the mulberry leaf extract (1-DNJ) has the potential to inhibit *Colletotrichum* spp. and enhance the chilli seed quality. Hence, 2% mulberry leaf extract (1-DNJ) could be adopted as a coating formulation for disease infected chilli seed.

Keywords: Anthracnose disease, 1-deoxynomyjirimycin, *Colletotrichum* spp., *Morus alba* L. extract, seed coating

Chilli pepper is one of the important commercial crops that are being cultivated and consumed all over the world. There are around 400 different varieties of chilies cultivated and commercialised globally. The most popular variety is *Capsicum annuum* L. (Chaudary et al. 2006). However, the chilli crop is always prone to pest and disease attack. There are many diseases that affect chilli plants and cause major yield losses. The fungal diseases that commonly affect chilli crops are anthracnose, cercospora (frogeye) leaf spot, downy mildew, fusarium stem rot, fusarium wilt, phytophthora blight, and powdery mildew (Hussain and Abid 2011). One of the most difficult diseases to control even by chemical application is anthracnose. Anthracnose disease has been reported to be a major constraint in chilli production in tropical and subtropical countries causing huge losses.

Anthracnose is a disease caused by various species of *Colletotrichum*, including *Colletotrichum capsici,* and *Colletotrichum gloeosporioides* (Mishra et al. 2019). The seed is very important for crop production as around the world over 80% of crops are propagated from seed. Previous studies reported that even a small infection in a seed is sufficient to trigger epidemics. Chigoziri and Ekefan (2013) revealed that fungi *C. capsici* and *C. gloeosporioides* are causal agents of fungus disease and persist on the infected seeds for long period of time. If the seeds are sown for the next season, they will lead to the reduction of seed germination and crop production due to severe seed fungal infection. These fungi infect the chilli crop through spore germination, appressorium formation, and host penetration (Motukuri 2017). After infection, the pathogen proliferates rapidly, causing symptoms such as

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dark sunken lesions on fruit surfaces and defoliation. The disease usually affects all plant parts, including leaves, stems, seeds, and fruits, causing yield loss. Studies have indicated that yield losses due to anthracnose can range from 30 - 60%, depending on its severity and environmental conditions (Athira et al. 2021; Saxena et al. 2016). There are many chemicals or fungicides used to control the anthracnose disease but the efficacy of controlling the infection is reducing over time due to the development of resistance. *Colletotrichum* species have shown resistance to many fungicides due to repeated application (Choudhary et al. 2013). Not only does resistance development among the fungal population become a problem, overuse of the fungicides also causes environment pollution. There is a need for an alternative and eco-friendly solution to reduce the infection of the anthracnose disease starting from the chilli seeds.

Natural compounds from plant extracts potentially can be used to manage fungal diseases in agricultural crops. One of the plants that can be found in abundance is mulberry. Mulberry leaves (*Morus alba* L.) are cultivated widely in Asian countries, and are stated to have antimicrobial, antioxidant, and antihyperglycemic properties (Naowaboot et al. 2009). Mulberry leaves have various phytochemicals such as flavonoids, phenolic acids, and alkaloids, which exhibit potent antimicrobial properties. Mulberry leaf extract could be used as a coating for diseased chilli seed to control anthracnose disease because it has 1-Deoxynojirimycin (1-DNJ). It is an iminosugar derived from mulberry leaves and exhibits notable antimicrobial and antifungal activities. The compound functions by inhibiting glycosidases, enzymes essential for the survival and virulence of various microbial and fungal pathogens (Kim et al. 2004). Coating seed with mulberry leaf extract could potentially suppress fungal related diseases. Seed coating is a sustainable way to improve the seed quality, suppress fungi, and enhance seed shelf life. Thus, seed coating can be summarised as an approach for seed treatment.

There is a lack of documented work on coating by mulberry leaf extract to enhance seed quality by suppressing the fungus that causes anthracnose disease. Hence, there is a need to assess the efficacy of this natural anti-fungal compound on chilli seed germination and seedling growth. Therefore, the objectives of this study were to: (i) extract mulberry leaf with 1-DNJ active compound, and (ii) to test the efficacy of the seed coating with mulberry leaf extract with 1-DNJ on its germination rate and growth performance against pathogens that cause anthracnose disease. Through this study, sustainable strategies for anthracnose control in chili cultivation by using seed coating method could be revealed.

Materials and methods

Mulberry leaf extraction and purification of 1-DNJ

Mulberry leaves were collected from Universiti Malaysia Kelantan, Kelantan, Malaysia. The leaves were washed thoroughly to remove dirt and other contaminants, and then oven dried at 40°C for 72 hours. The leaves were then ground into a fine powder using an electrical blender. A 100 g-portion of the powdered mulberry leaves was mixed with ethanol in a ratio of 1:10 (mulberry leaf powder to ethanol). The container was tightly sealed and shaken in an orbital shaker for thorough mixing. The mixture was allowed to steep for 48 hours. Then the mixture was filtered using filter paper to separate the solid residue from the liquid extract. The resulting liquid was collected, and solvents were separated using rotary evaporation. A chromatography column was set up with a stationary phase of silica gel. The column was prepared by packing the silica gel and then equilibrated with a solvent mixture, hexane, methanol, and water. The concentrated crude extract was carefully loaded onto the column and the column was eluted using a solvent system optimised for the separation of 1-DNJ

from other compounds in the extract. The fractions were collected, and the separation was completed by using thin-layer chromatography (TLC). The collected fractions were analysed using TLC and those containing 1-DNJ were identified. The fractions containing 1-DNJ were pooled. The purified 1-DNJ fraction was then concentrated, and then the 1-DNJ fraction was further dried using freeze-drying before further use.

Chilli seed coating and germination test in a controlled environment

The infected chilli fruit (var Kulai) was sampled from a chilli farm in Kelantan. The chilli seeds used in this study were collected from anthracnose infected plants. Coating solutions were prepared following Yang et al. (2014) and Sabaghi et al. (2015). The diseased chilli seeds were coated with 1%, 2%, 3%, and 4% 1-DNJ mulberry leaf extract (Biel-Parzymieso 2020; Insang et al. 2022). A positive control treatment with 1% thiram fungicide was also applied and diseased chilli with neither 1-DNJ nor thiram served as a negative control. For all treatments diseased chilli seed was also coated with 2% glycerine. The coated chilli seed was air dried for 24 hours before storage in a sterile can. The treatments are listed in Table 1.

Table 1: Diseased chilli seed coating treatments

Treatments	Details
C	Diseased chilli seed + 2% glycerin
T	Diseased chilli seed + 1% thiram + 2% glycerin
M1	Diseased chilli seed + 1% mulberry leaf extract + 2% glycerin
M2	Diseased chilli seed + 2% mulberry leaf extract + 2% glycerin
M3	Diseased chilli seed + 3% mulberry leaf extract + 2% glycerin
M4	Diseased chilli seed + 4% mulberry leaf extract + 2% glycerin

The leaf-extract-coated and uncoated chilli seeds were tested for germination in glass petri plates with 15 diseased chilli seed of each treatment in each petri dish. There were three replicates. To moisten the filter paper before placement of the seeds 5 ml of distilled water was added. The petri plates were sealed by using parafilm after the placement of seeds. The sealed petri plates were arranged in growth chamber with a temperature of 29 ^{0}C.

Isolation of *Colletotrichum* spp. and coated seed germination testing in PDA media

The infected tissue was cut into small pieces and surface sterilised with 0.1% sodium hypochlorite solution for 30 seconds followed by washing with sterile distilled water 2-3 times. The sterilised potato dextrose agar (PDA) medium was poured into the sterilised petri plates and allowed to solidify. Sterilised infected pieces of chilli fruit rot samples were placed in three places at equal distances and incubated at 28^{0}C for 10 days. The growing young fungal hyphal tips were transferred to the sterile plates containing PDA medium aseptically in order to obtain pure cultures by following the hyphal tip method (Sinclair and Dhingra 2017). The pure cultures of all the five isolates were maintained in PDA slants. Further subculturing was done from the slants and after 10 days of incubation, the plates were observed for the growth and morphological characters of the isolates such as colour of mycelium, radial mycelial growth, shape, and colour of the conidia to obtain *Colletotrichum* spp. (Qiao et al. 2021).

The laboratory experiment to observe the germination of coated and uncoated chilli seed when in the presence of the *Colletotrichum* spp. fungus was conducted in PDA media. The

PDA media inoculated with *Colletotrichum* spp. were sown with 15 seeds according to the treatments listed in Table 1. The experiment was conducted in laminar flow hood to maintain the aseptic conditions. The PDA media was immediately sealed with parafilm to avoid contamination. Later, the PDA media was arranged in an incubator for seven days at 29^0C and observed for the germination rate. The data of seed germination, root length, and shoot height were collected after 7 days.

Pot experiment

The coated and uncoated diseased chilli seeds with mulberry leaf extract were further tested in a pot experiment to evaluate their growth performance. A total of 18 pots (12 × 12 cm) were filled with soil as a medium of planting. Each pot was sown with three seeds of coated and uncoated chilli according to the treatments. All the pots were arranged in a completely randomised design with three replications at the nursery of Universiti Malaysia Kelantan. The plants were checked and watered daily. The chilli plants were harvested 14 days after sowing and root length, shoot fresh weight, and shoot height were measured.

Data analysis

Both experiments were arranged in a completely randomised design with three replicates of each treatment. The effect of different treatments was subjected to one-way analysis of variance. Significant differences among treatments were separated by Tukey's HSD test and considered significant at $P \leq 0.05$. Statistical analysis for all the data was performed using SPSS software version 24.0 (SPSS Inc, US).

Results and discussion

Colletotrichum spp. identification

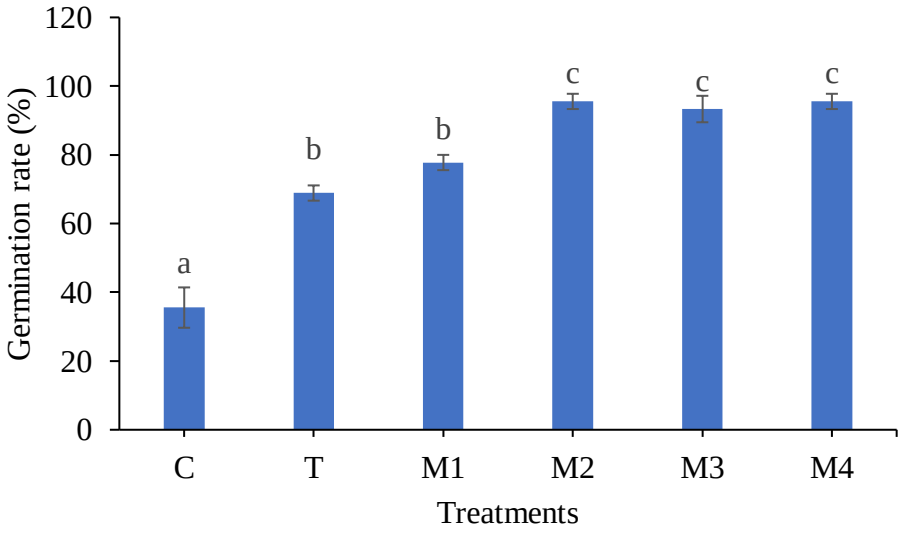
The identification of *Colletotrichum* spp. was conducted in general by observing the morphological characteristics. Pongpisutta et al. (2013) stated that the identification of *Colletotrichum* species could be based on the morphological characteristics such as spore mass, fungal colony, colour, shape and size of spore, growth temperature and growth rate. In this study, the colony colour varied from whitish to greyish to pinkish with cottony aerial mycelium. Conidia produced varied from fusiform with obtuse to slightly rounded ends to sometimes oblong with cylindrical shape. The identified features are proven to be the *Colletotrichum* spp. characteristics. The characteristics of *Colletotrichum* spp. found in this study are almost similar to the findings of Kamdoum et al. (2016). However, the exact species of *Colletotrichum* spp. could not be identified as either *C. gloeosporiodes* or *C. acutatum* or others because their structure, size and shape are almost similar. Hence, in this study, *Colletotrichum* spp. was used in general because accurate identification by using molecular technology is needed for confirmation of the exact fungal species.

Germination rate of coated and uncoated diseased chilli seeds

The chilli seed that was collected from anthracnose-infected plants was coated with mulberry leaf extract (1-DNJ) in order to improve the germination rate and their growth. The 1-DNJ compound is proven to have excellent antimicrobial properties. The ethanol extracted mulberry leaf had significantly inhibited the growth of *Pseudomonas aeruginosa* and *Escherichia coli* microorganism (Adithya et al. 2012). The ethanol extract exhibited stronger antimicrobial activity than distilled water extracts (Emniyet and Acci 2015). This proves that mulberry leaf extract that contains 1-DNJ compound could have a strong antifungal effect against *Colletotrichum* spp. This antifungal property is crucial to enhance the germination of diseased chilli seed.

The germination rate determines the viability of the diseased chilli seed. Seed germination percentage is the number of

normal and healthy seedlings under optimum conditions. Figure 1 shows that the germination percentage of seed in treatments coated with 2, 3, and 4% of mulberry leaf extract was significantly higher than the seeds coated with glycerin only, thiram and 1% mulberry leaf extract. The germination percentage in treatments M2, M3, and M4 was over 80%. This germination percentage of over 80% shows a good indication of seed viability (Lian et al. 2020). Mulberry leaf extract coatings of 2% or more significantly increased diseased chilli seed germination over the standard thiram fungicide which is used commercially. This could be due to the active compound of 1-DNJ that present in the mulberry leaf extract, which has antimicrobial properties. This gives an indication that the 1-DNJ compound is able to suppress the fungal growth on the chilli seed by seed coating. It has been stated that biopriming of chilli seed significantly reduces the infestation of anthracnose disease caused by *Colletotrichum capsici* (Athira et al. 2021).



C: diseased chilli seed + 2% Glycerin. T: diseased chilli seed + 1% thiram + 2% Glycerin. M1: diseased chilli seed + 1% mulberry leaf extract + 2% glycerin. M2: diseased chilli seed + 2% mulberry leaf extract + 2% glycerin. M3: diseased chilli seed + 3% mulberry leaf extract + 2% glycerin. M4: diseased chilli seed + 4% mulberry leaf extract + 2% glycerin.

Figure 1: The effect of mulberry leaf extract coating on chilli seed germination. Mean values with different letter(s) indicate significant difference between treatments by Tukey's test at $P \leq 0.05$. Bars represent the mean values ± SE. The overall pooled SE of the treatments is 5.31.

Germination and chilli seed growth in PDA media inoculated with Colletotrichum spp.

The effect of coated and uncoated chilli seed on germination rate, root length, and shoot height is shown in Table 2. The germination rate of chilli seed coated with mulberry leaf extract was significantly higher than treatments C and T. Similar observation observed for the growth of chilli plant root length. The roots of plants from seeds coated with the mulberry leaf extracts (M1, M2, M3, and M4) were significantly longer than seed in treatment control and with thiram. The heights of shoots of plants developing from coated seeds is significantly taller in treatments M2, M3, and M4 compared to C and T. Coating chili seed with 2% or more mulberry leaf extract (1-DNJ) showed a good indication of *Colletotrichum* spp. suppression and indicates that it has a potential to be antifungal agent.

Even when seeds were germinated in PDA inoculated with the fungus, the germination of coated chilli seed and subsequent seedling growth were not affected. This showed that mulberry leaf extract with 1-DNJ compound suppressed the activity of *Colletotrichum* spp. The result was in accordance with the previous study conducted by Kwon et al. (2019) who stated that mulberry plant extract significantly exerted the highest inhibitory activity against the mycelial growth of *Alternaria alternata* and *Fusarium* sp. fungus. Seed coating with mulberry leaf extract (1-DNJ) had shown a positive effect in *Colletotrichum* spp. inhibition, and this is in agreement with Gowtham et al. (2018), who found that seed coating in chilli resulted in significant protection of 71% against anthracnose caused by *Colletotrichum truncatum*.

Table 2: The effect of mulberry leaf coating on chilli seed germination, and chilli plant growth (root length and shoot height) on 14th days after planting

Treatments	Germination rate (%)	Root length (cm)	Shoot height (cm)
C	28.9[a]	0.4[a]	2.7[a]
T	60.0[b]	1.0[b]	3.7[b]
M1	75.6[c]	1.4[c]	4.1[bc]
M2	84.4[cd]	1.9[d]	5.0[d]
M3	88.9[d]	1.9[d]	4.7[cd]
M4	93.3[d]	1.9[d]	4.9[d]
Pooled SE	5.43	0.14	0.21

Mean values within column with different letter(s) indicate significant differences between treatments by Tukey's test at $P \leq 0.05$. Columns represent the mean values ± SE. C: diseased chilli seed + 2% Glycerin. T: diseased chilli seed + 1% thiram + 2% Glycerin M1: diseased chilli seed + 1% mulberry leaf extract + 2% glycerin. M2: diseased chilli seed + 2% mulberry leaf extract + 2% glycerin. M3: diseased chilli seed + 3% mulberry leaf extract + 2% glycerin. M4: diseased chilli seed + 4% mulberry leaf extract + 2% glycerin.

Coated and uncoated chilli seed growth in pot experiment

Similar observations were found in the growth performance of coated and uncoated chilli seed in the pot experiment under nursery environment. The measurements of seedling growth are presented in terms of root and shoot lengths and shoot fresh weight in Figures 2, 3 and 4. The root length and shoot height were significantly improved in treatments with 2% or more mulberry seed extract (M2, M3, and M4) compared to the other treatments. As for shoot fresh weight, only treatment M2 was significantly higher in comparison to C and T. A previous study found that mulberry leaf extract had promoted the root and shoot dry matter production by greater than 87%, and this might be related to the presence of phytotoxins in the mulberry extract (Haq et al. 2010). Similarly, mulberry leaf water extract promoted the germination and growth of pea and broad bean plants (Mughal 2000). Further, from the visual observation, the plant growth from coated chilli seed was excellently good and not wilted compared to the control and thiram treatments. The increased length of radicle indicates a good growth of chilli plant, where the roots are healthy to absorb nutrients from the soil and applied fertilisers. This is in agreement with research conducted by Selvarajh et al. (2017, 2018, 2023) who stated that root growth is important for absorbing nutrients. This result implies that treatments of mulberry leaf extract coating (1-DNJ) can maintain healthy seed germination even under conditions of fungal infection. The seed survival rate is higher due to the inhibitory effect exerted by the 1-DNJ compound present in the mulberry leaf extract on the fungus. It has the potential to inhibit the mycelium and spore germination of *Colletotrichum* spp., in

which it reduces the overall seed infection and enhance chilli seed quality. It has been shown that the decrease of seed infection percentage is directly related to high seed germination due to the effective seed treatment (Ahmad et al. 2022).

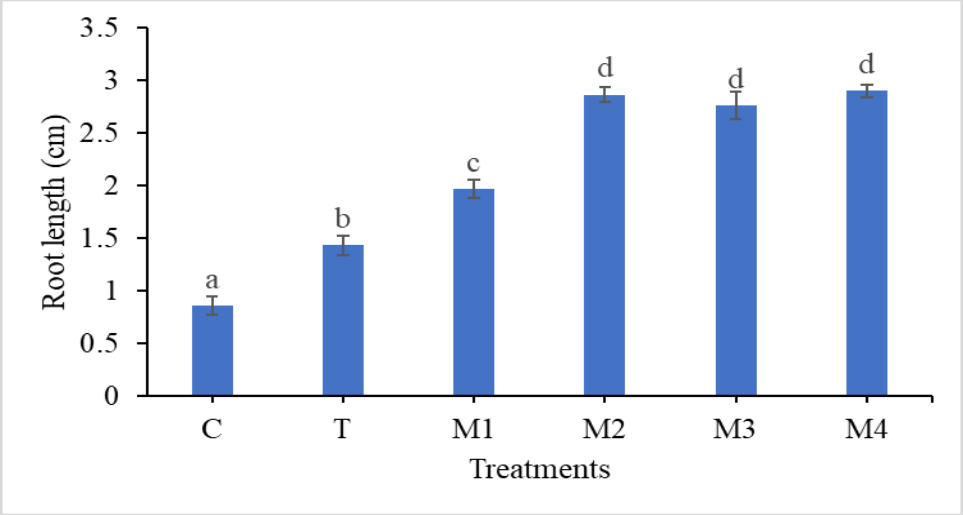


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Figure 2: The effect of mulberry leaf extract coating on root length of chilli plant 14 days after planting in the pot experiment. Mean values with different letter(s) indicate significant difference between treatments by Tukey's test at P ≤ 0.05. Bars represent the mean values ± SE. The overall pooled SE is 0.19.

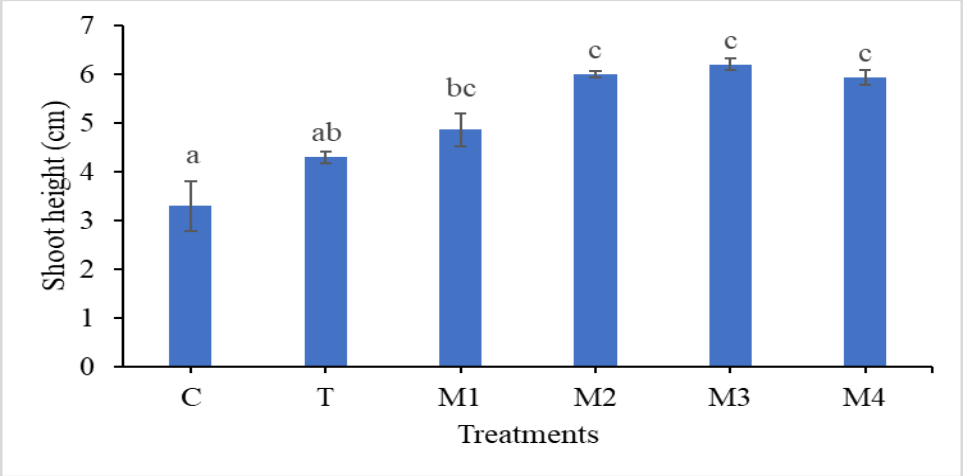

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Figure 3: The effect of mulberry leaf extract coating on shoot height of chilli plant 14 days after planting in pot experiment. Mean values with different letter(s) indicate significant difference between treatments by Tukey's test at P ≤ 0.05. The overall pooled SE is 0.28.

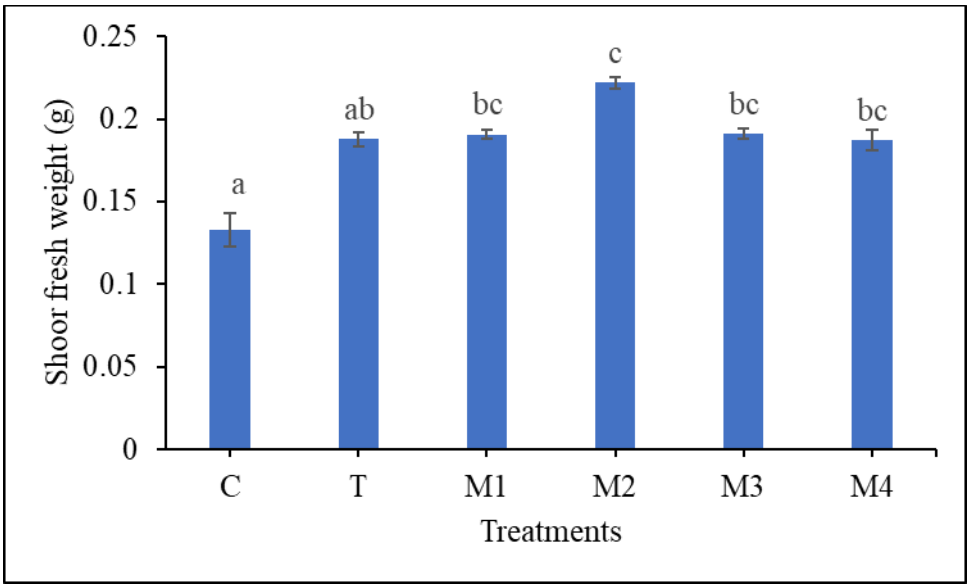

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Figure 4: The effect of mulberry leaf extract coating on shoot fresh weight of chilli plant on 14th days planting in pot experiment. Mean values with different letter(s) indicate significant difference between treatments by Tukey's test at P ≤ 0.05. Bars represent the mean values ± SE. The overall pooled SE is 0.01.

Conclusion

Mulberry leaf extract coating with active compound 1-DNJ enhances the germination and seedling growth of anthracnose infected chilli seed. The seed coating significantly suppressed the *Colletotrichum* spp. in PDA media and increased the chilli seed germination, root length and shoot length. Similar observations were observed in the pot experiment. Hence 2% mulberry leaf extract could be adopted as a formula of diseased chilli seed coating to inhibit the activity of *Colletotrichum* spp. However, further comprehensive research is needed to test the validity of data by incorporating a field study for three cycles.

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