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Research Article

Anatomical Alterations and Management Strategies of *Diplocarpon rosae* Infection in *Rosa centifolia*

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ABSTRACT

Black spot caused by *Diplocarpon rosae* is a serious issue of rose. Current study was conceived to assess antifungal potency of five different phyto-extracts including Cinnamon, Turmeric, Black pepper, Ginger and Aloe vera against *D. rosae* under laboratory and in planta conditions. All the phyto-extracts were evaluated at three concentrations under completely randomized design (CRD) with three replications of each treatments under laboratory conditions. However, the most effective treatments (cinnamon and turmeric) under lab conditions were further examined in planta against black spot of rose. The results revealed that, cinnamon was the most effective treatment against D. rosae under lab conditions with least mycelial growth (4.61 mm) followed by Turmeric (6.50 mm), Black pepper (7.24 mm), Ginger (8.06 mm) and Aloe vera (7.60 mm). While, the combined application of cinnamon and turmeric exhibited least disease incidence (32.63%) under *in-vivo* conditions. Being an airborne pathogen, *D. rosae* affects the anatomy of the infected tissues. So, the contemporary study was designed to investigate the anatomical alterations including Midrib thickness (MIT) (µm), Vascular bundle area (VBA) (µm^2), Adaxial epidermal thickness (ADET) (µm), Abaxial epidermal thickness (ABET) (µm) and Parenchymatous cell area (PCA) (µm^2) of rose leaf cells infected with *D. rosae*. The analysis was comprised of three main targets such as among healthy & infected, treated & untreated and at different inoculation stages of rose leaf cells. The samples of rose plants were gathered from Horticulture Area of UAF. Following typical anatomical procedures, the leaf samples were examined under digital compound microscope. All the observations exposed that there was no abnormality found in healthy leaf sample, while the infected sample showed an expanded MIT, ruptured ABET, sclerified ADET, decreased VBA and larger PCAs. Furthermore, at different inoculation stages the MIT was found expanded in all three stages, PCAs began increased in size and VBA become shrunk in second stage of inoculation. While after treatment with cinnamon and turmeric alone and in combination, the anatomical observation showed that combination of cinnamon and turmeric exhibited positive effect on normalizing the measurements of investigated parameters, whereas individual application of turmeric only reduced PCAs to normal size.

Keywords: Black-spot, Cinnamon, Midrib thickness, Phyto-extracts, Vascular bundle area.

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INTRODUCTION

Rose has been known by its beauty and fragrance since a long time (Mileva et al.

2021). Rose belongs to family Rosaceae that comprises of about 18000 cultivars, 200 species and it is native to North America, Northwest Africa, Asia, and Europe (Sazzad et al. 2022; Quratulain et al. 2015). In this genus, 8 rose species viz *R. chinensis, R. foetida, R. damascena, R. gigantea, R. moschata, R. gallica, R. wichuraiana, R. multiflora* entirely perform a major role in the evolution of modern garden roses (Batool et al. 2018; Gaurav et al. 2022). Among all, *Rosa centifolia* is known to be a very famous oil bearing rose specie with pink colored fragrant flowers. Its flowers contain bright pink colored petals with a calyx tube having reddish hairs. In Pakistan, *R. centifolia* continuously produces flowers during whole year and even in very warmer conditions when other related species stop flowering. This long-term flowering is beneficial for farmers in terms of price and regular petal supply for the production of essential oils (Akhtar et al. 2019; Martínez et al. 2020).

India is one of the biggest rose cultivars in the world with an area of about 28130 ha, next one is China having an area of 14316 ha and then Ecuador with 4073 ha. Major rose exporters are Netherland, Ethiopia and Kenya having a cultivation area of 775.5 ha, 299.5 ha and 136.3 ha respectively. In Pakistan, rose is cultivated on an area of about 15000 acres (Shah et al. 2021). *Rosa centifolia* has gained much economic importance due to the production of commercially valuable products like rose water, rose oil and perfumes. It also has a great medicinal importance due to its antibacterial, anticancer, antimicrobial, antioxidant and geno-protective activities (Mileva et al. 2021).

Rose species are the most adversely affected by black spot disease. Black spot disease was discovered in Europe for the first time in 19th century, later in Germany, France, England, Netherland and Belgium. This disease caused severe reduction in rose yield and also affects its growth rate. *Diplocarpon rosae* is the main causal agent of black spot disease in rose species that belongs to the order Helotiales and family Dermataceae, having both asexual and sexual stages termed as *D. rosae* Wolf and *Marssonina rosae* Lind respectively. This fungus has a worldwide distribution and a main problem among all garden roses (Debener 2019). As a result of this disease, purple to black colored circular or irregular spots are formed on the upper as well as lower surface of leaves. Due to this infection leaves become yellow and start falling at their early stages (Lopez et al. 2020). *Diplocarpon rosae* mainly propagated through a bicellular conidium that spread through rain drops, rodents and insects. Mechanism of fungal penetration starts with the formation of germ tube upon conidial germination having an appressorium at its end. A penetration peg is formed at the end of appressorium in order to penetrate the host cuticle and as a result of this penetration a subcuticular vesicle is formed which give rise to the formation of both subcuticular and intercellular hyphae that further penetrates into the epidermal and parenchymatous tissues by forming specific functional structures called haustoria (Debener 2019).

Plant diseases are mainly managed by using chemical pesticides which impose highly hazardous effects on humans, animals as well as surrounding environment, that's why the researchers are finding the new ways of plant disease management having no or limited hazardous or residual effects on human and animal health. However, phyto-extracts are also one of the green plant disease management practices, which are safe, non-toxic, easily degradable with an efficient mode of action due to their antioxidant and antimicrobial activities (Qaisar et al. 2023; Simon et al. 2016). These are being used as a green-fungicides as an alternative to synthetic chemicals all over the world to avoid the harmful effects of black spot disease caused by *D. rosae* (Sabarinathan et al. 2019). Anatomical study of any pathogenic infection could further enlighten the way to find out numbers of alterations in cellular anatomy, thus the current study was also planned to find out the structural alterations caused by *D. rosae* in rose plants through microscopy before and after management practices, to ensure the effectiveness of phyto-extracts.

MATERIALS AND METHODS

Isolation, Purification and Identification of *Diplocarpon rosae*

Diseased leaf samples having circular purple to black colored spots and lesions were observed and collected from horticulture field area at University of Agriculture, Faisalabad and brought to laboratory for the isolation of pathogen. The diseased leaves were rinsed with tap water and then cut into small pieces having both healthy and diseased portion. The sample pieces were disinfected by immersing in 1% NaOCl solution for 30 seconds, then washed three times with distilled water until complete removal of Sodium hypochlorite. Then, sample pieces were dried using sterilized tissue paper and placed on petri plates containing autoclaved potato dextrose agar (PDA) media. These plates were wrapped and incubated in an incubator (GEN2BOD) at 25^{o}C. For purification, single hyphal tip method was used in which single mycelium was picked and placed on the fresh PDA culture plates using sterilized inoculating needles. The pathogen was then identified on the basis of morphological characters including colony color, shape and spore's structure (Matte et al. 2022).

Pathogenicity Test

In order to confirm the pathogen, pathogenicity test was performed under field conditions by artificially inoculating the rose plants with spore suspension. Fungal spore suspension was prepared and adjusted to 1×10^5 conidia per ml by using a haemocytometer. The symptoms were appeared within 7 days and these were same as the black spot disease caused by *Diplocarpon rosae*. Then, the pathogen was re-isolated from diseased leaf samples. Both microscopic and colony characters of this re-isolated fungus were similar to the parent culture of *D. rosae* (Matte et al. 2022).

***In-vitro* Management of *Diplocarpon rosae* Through Phyto-Extracts**

For *in-vitro* experiment, powder of *Curcuma longa*, rhizomes of *Zingiber officinale*, leaves of *Aloe vera*, fruits of *Piper nigrum* and quills of *Cinnamomum verum* were bought from local market of Faisalabad and taken to the Plant Pathogen-Interaction Laboratory, UAF. All the washable materials were washed under tap water in order to remove dirt particles and placed in open air environment for drying. After drying, all the plant materials were weighed as 100 grams and blended separately using an electronic blender to obtain aqueous solutions. After that, the aqueous solutions were transferred to clean flasks, where mouth of flasks were covered with aluminum foil and placed on shaker for whole night. Finally the material was filtered using sterilized muslin cloth. Three different concentrations i.e. 10%, 20% and 30% were prepared by adding 10, 20 and 30 ml of aqueous solutions directly in 100 mL autoclaved and hot PDA media. After mixing, the amended PDA media was poured in sterilized petri plates and kept for solidification. The petri dishes were then inoculated with 5mm discs of fresh culture of *Diplocarpon rosae* aseptically. Petri plates contained un-amended media were used as control treatments. All the petri plates were wrapped and incubated at the temperature of 25°C. The experiment was conducted under completely randomized design (CRD) with three replications of each treatment to minimize error.

***In-vivo* Management of *Diplocarpon rosae* Through Phyto-Extracts**

Among all phyto-extracts, investigated under laboratory conditions two most effective phyto-extracts such as Cinnamon (*Cinnamomum verum*) and Turmeric (*Curcuma longa*) were used alone and in combination against black spot disease of rose under *in-vivo* conditions. Firstly, already grown rose plants were inoculated with fungal suspension using hand sprayer, after that, the extracts were applied in the same manner. The whole experiment was conducted under randomized complete block design (RCBD) having three replications of each treatment whereas each replication contained 3 plants. The control treatment were kept untreated. After one week of application, the data was recorded by using the formula given by (Mansha et al. 2021):

$$Disease, incidence = \frac{No\ of, infected\ plants}{No, of\ total\ plants\ assessed} \times 100$$

Anatomical Analysis

Three types of rose leaf samples were collected for anatomical analysis i.e. healthy, infected with *Diplocarpon rosae* and infected but treated with cinnamon, turmeric and their combination samples. Leaf sections of about one cm were cut from the central area of leaf along with midrib by free hand sectioning. These sections were fixed by using formalin acetic alcohol (FAA) and then transferred to acetic alcohol solution for prolonged preservation. The sections were then dehydrated using a series of 30%, 50% and 90% alcoholic solutions. After dehydration, these were stained using safranine (for staining of lignified tissues) and fast green dyes respectively. Finally, after washing with absolute alcohol (100%) for three times these were then transferred to xylene for complete removal of dyes. Then these transverse sections were fixed on a glass slide using Canada balsam and observed under camera-equipped digital compound microscope (MT4300-LV-HD, Meiji Techno, Japan). Leaf parameters i.e. Midrib thickness (MIT) (µm), Vascular bundle area (VBA) (μm^2), Adaxial epidermal thickness (ADET) (µm), Abaxial epidermal thickness (ABET) (µm) and Parenchymatous cell area (PCA) (μm^2) were studied.

Statistical analysis

The recorded data of laboratory and field experiments was statistically analyzed by using statistical software (Statistics 8.1). Whereas, Complete Randomized Design (CRD) for laboratory and Randomized Complete Block Design (RCBD) was used for field experiments (Steel, 1997). Using LSD at P=0.05% probability level treatment means were separated. The impact of disease and different treatments on anatomical factors were determined using principal component analysis (PCA).

RESULTS

Isolation, Purification and Morphological Identification of *D. rosae*

The diseased leaf samples were subjected to isolation of pathogen. With the help of literature and morphological characters, *Diplocarpon rosae* was identified. Typical morphological characters of *D. rosae* including grayish black colored mycelial growth with binucleate, two celled, aseptate and colorless conidia were observed under microscopy (Figure 1).

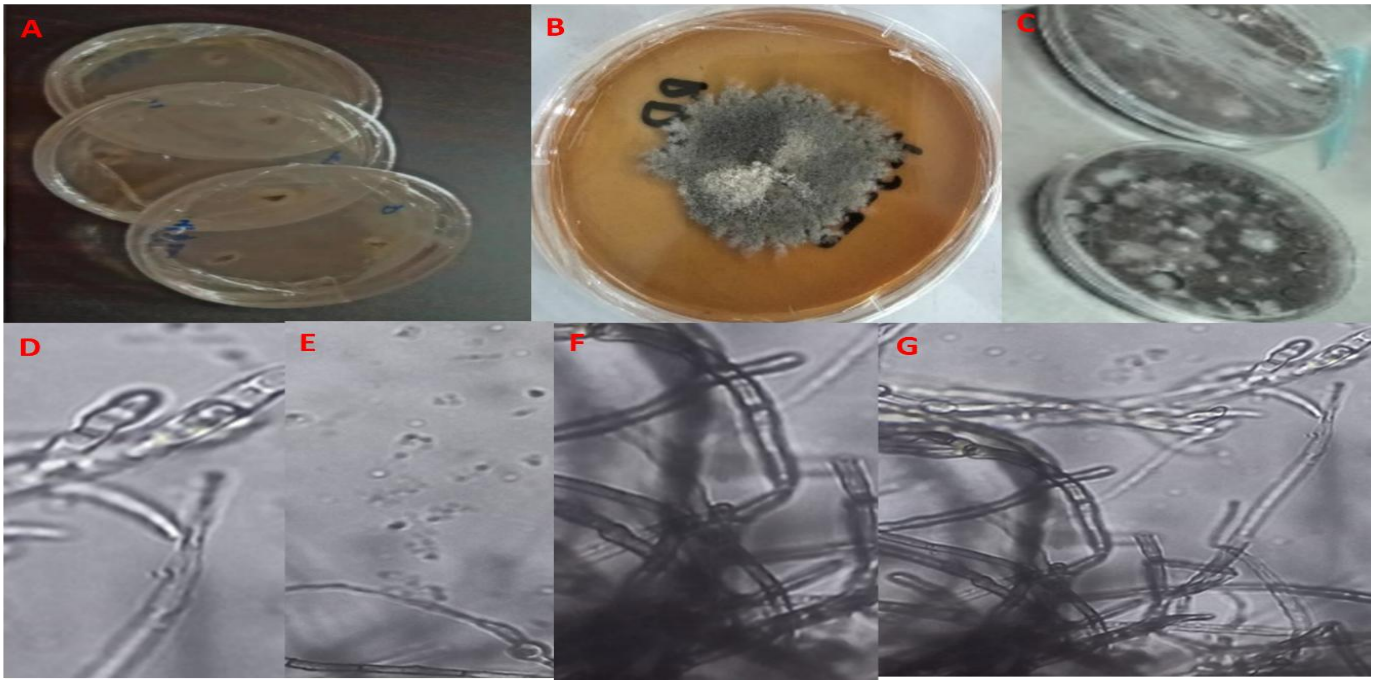

Figure 1. Pictorial illustration of (a) petri plates showing initial growth of isolation (b & c) purified culture of *D. rosae* after three and eight days (d, e, f & g) elaborating microscopic observance of conidiophores, mass of conidia and acervuli.

Pathogenicity Test

After inoculating the pots with spore suspension, symptoms of black spot disease were observed within 1 week. In the early stage small, purplish and round shaped spots were start appearing but on the onset of maturity, large, blackish spots with irregular margins was observed. As a result of this disease, symptoms of chlorosis in leaves were also observed (Figure 2).

Figure 2. Symptoms of black spot of rose after artificial inoculation.

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In-vitro Evaluation of Phyto-Extracts Against *Diplocarpon rosae* Causing Black Spot Disease of Rose

Mean comparison of all the phyto-extracts indicated the significant antifungal potential of Cinnamon, where minimum fungal growth (4.61 mm) was recorded, followed by Turmeric (6.50 mm), Black pepper (7.24 mm), Ginger (8.06 mm) and Aloe vera (7.60 mm) (Figure 3a). Analysis of interaction between treatments and concentrations exposed that at all three concentrations i.e. 10%, 20% and 30% cinnamon expressed minimum mycelial growth (5.64, 5.10 and 3.10 mm) respectively, whereas Turmeric (8.05, 6.83 and 4.61 mm), Black pepper (7.70, 7.22 and 6.80 mm) Ginger (8.55, 8.00 and 7.64 mm) and Aloe vera (9.22, 7.89 and 5.70 mm) also showed reduction in mycelial growth with increment of phyto-extract's concentration (Figure 3b). The interaction between treatments and time intervals showed that the maximum mycelial growth was expressed by the application of Aloe vera (6.48, 7.84 and 8.48 mm) at 1st, 2nd and 3rd day of application and exposed it as least significant treatment, however, Cinnamon application exhibited minimum mycelial growth (3.92, 4.58 and 5.33 mm) at the same days of incubation and reflected it as potential treatment against *D. rosae* (Figure 3c).

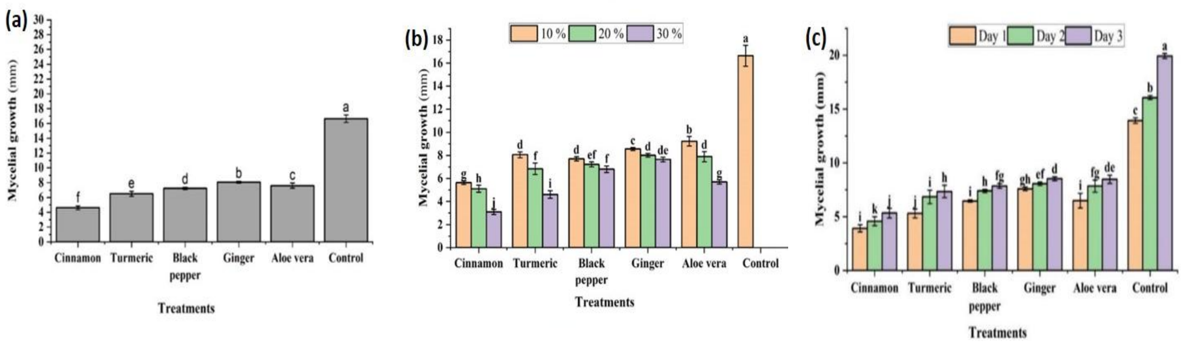

Figure 3. Assessment of efficacy of phyto-extracts against *Diplocarpon rosae* under *in-vitro* conditions, where (a) refers mean mycelial growth of *D. rosae* in response to different phyto-extracts, (b) is the impact of interaction between treatments and concentration on growth of pathogen and (c) is the effect of interaction between treatments and time interval on mycelial growth of pathogen.

In-vivo Evaluation of most Effective Phytoextract Individually and in Combination Against Black Spot of Rose caused by *D. rosae*

All the treatments showed significant control of disease. Among all the treatments, combination of Cinnamon + Turmeric showed minimum disease incidence (32.63%) followed by individual applications of Cinnamon (35.13%) and Turmeric (42.88%) (Figure 4a). While the interaction between treatment and days expressed that the combination of Cinnamon + Turmeric exhibited the positive reduction of disease incidence (26.66%, 33.83%, 37.41%) after 1st 2nd and 3rd week of application as compared to Cinnamon (31.16%, 35.83%, 38.41%) and Turmeric (37.16%, 42%, 49.5%) respectively (Figure 4b).

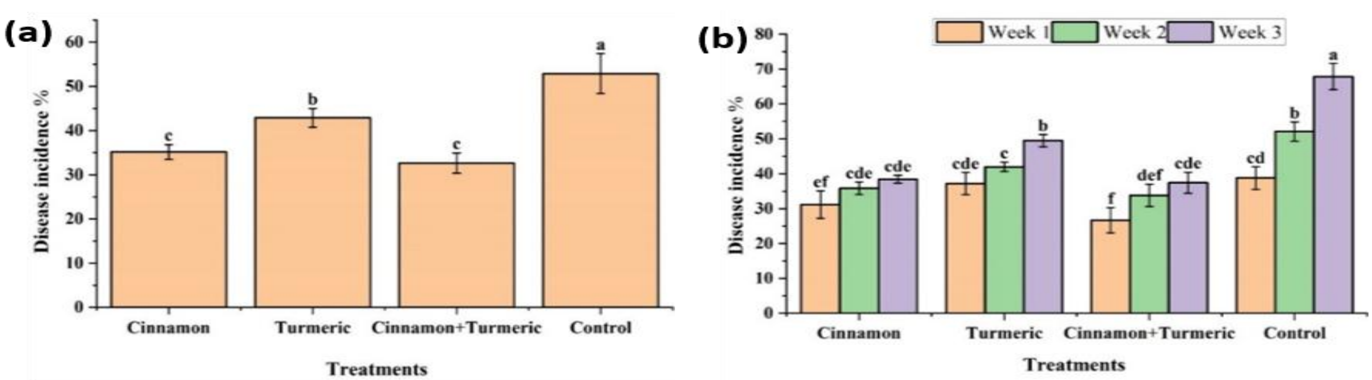

Figure 4. Assessment of potency of phyto-extracts against black spot of rose caused by *D. rosae* under *in-vivo* conditions. (a) is the mean disease incidence percentage after application of treatments and (b) is the impact of interaction between treatments and time interval on disease incidence percentage.

Anatomical Analysis

Anatomical study exposed that, all the investigated parameters including midrib thickness (1732.20 µm), adaxial epidermal cells (62.83 µm), abaxial epidermal cells (44.21 µm), vascular bundle area (2095796 µm^2) and parenchymatous cells (24925.4 µm^2) were in their normal position in healthy cells of *Rosa centifolia* leaves, while in a diseased leaf sample midrib thickness (2440.6 µm) increased, abaxial epidermis (61.77 µm) was observed to be ruptured and adaxial epidermis (99.32 µm) was sclerified, vascular bundle (1566059 µm^2) area was decreased and parenchymatous cells (105947.4 µm^2) become larger in size (Figure 5 & 6)**.**

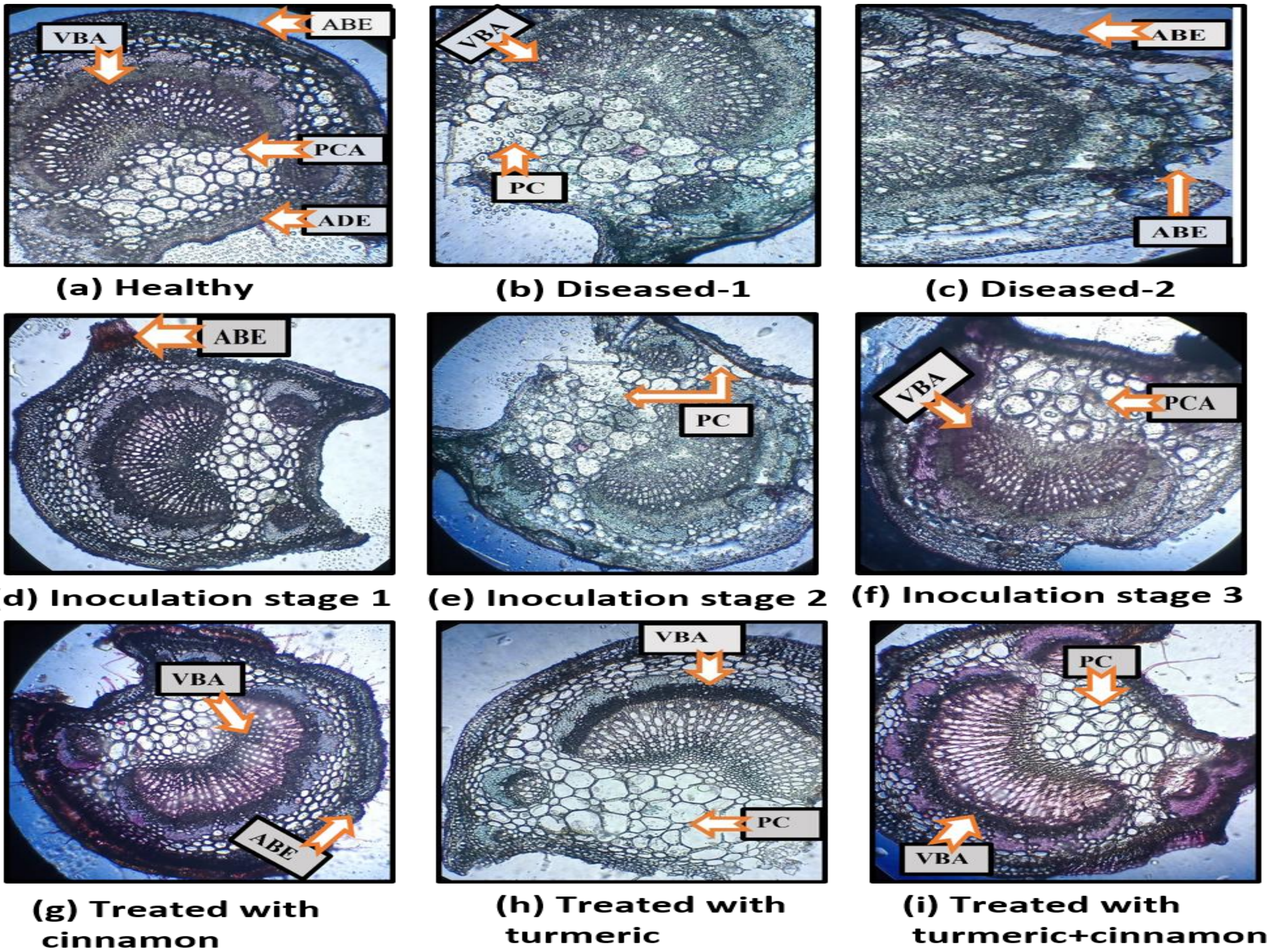

Figure 5. Pictorial illustration of anatomical alterations of rose leaf cells, where (a) is a healthy leaf with normal adaxial epidermis (ADE), abaxial epidermis (ABE), vascular bundles (VBA) and parenchymatous cells (PC), (b & c) diseased leaf cell showing shrunken vascular bundle area (VBA) and enlarged parenchymatous cells (PC) with ruptured and sclerified Abaxial epidermis (ABE). (d, e & f) are showing the inoculation stage wise anatomical alteration among diseased leaf cells, where in stage-1, abaxial epidermis (ABE) was ruptured due to fungal penetration, in stage-2 parenchymatous cells (PC) was found enlarged and in stage-3, large vascular bundle area (VBA) and parenchymatous cells (PC) with excessive sclerification was observed with reduced midrib. (g, h & i) elaborating that inoculated but cinnamon treated leaf showed normal vascular bundles with ruptured and sclerified adaxial epidermis (ADE), where turmeric treatment exhibited no effect on investigated parts of the inoculated cell. However, combine treatment of cinnamon and turmeric revealed normal vascular bundle area (VBA), healed parenchymatous cells (PC) and reduced midrib thickness.

Whereas, findings of different inoculation stages revealed that the midrib thickness was found increased in all three inoculation stages, additionally parenchymatous cells started increasing in size while the vascular bundle become shrunk in second stage of inoculation as shown in Figure 7. Furthermore, the findings after application of treatments (cinnamon, turmeric and combination of cinnamon and turmeric) disclosed that cinnamon reduced the size of midrib thickness (835.82 µm), adaxial epidermal cells (93.87 µm), abaxial epidermal cells (57.53 µm), vascular bundle area (1810712.64 µm^2) and parenchymatous cells (47009.41 µm^2) on infected leaf sample, while upon turmeric treatment, only parenchymatous cells (62023.82 µm^2) were decreased in their size. although the combination of turmeric and cinnamon effectively reduced the measurement of all the parameters to their normal size such as MIT (757.08 µm), ADET (76.91 µm), ABET (40.57 µm), VBA (2997005.87 µm^2) and PCA (22965.64 µm^2) (Figure 8).

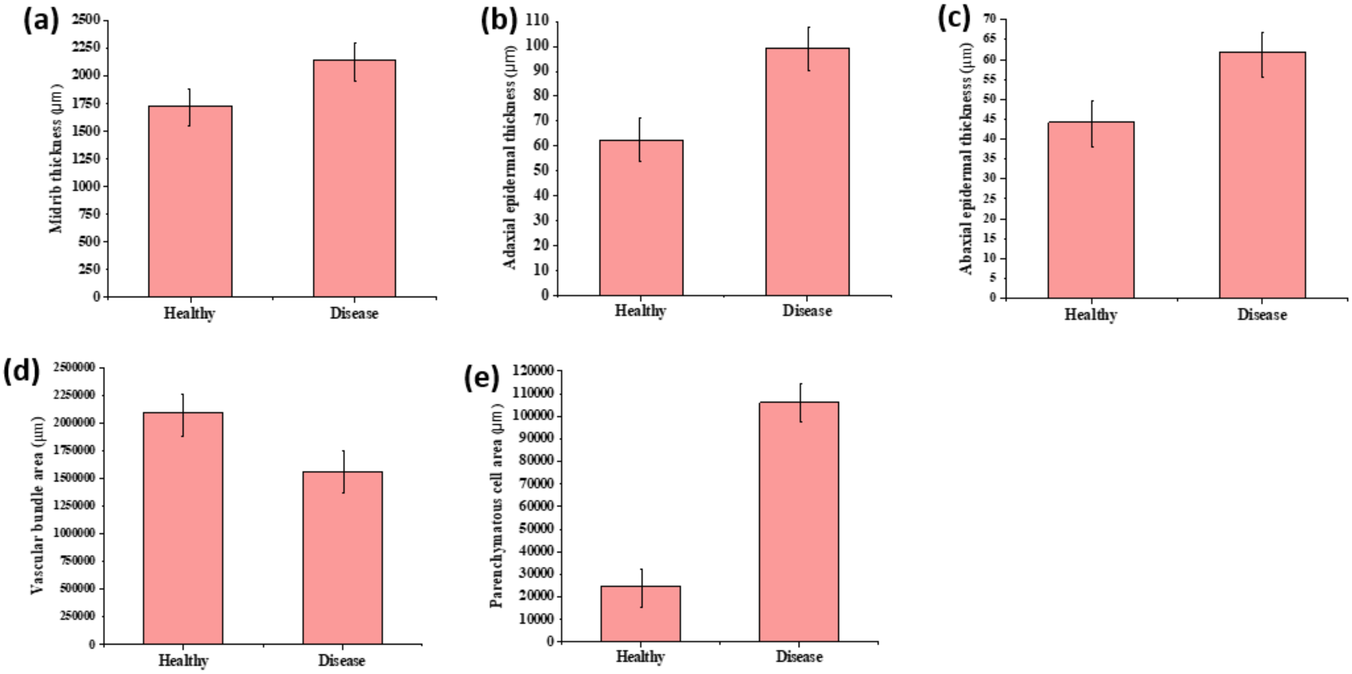

Figure 6. Estimation of (a) midrib thickness (b) adaxial epidermal thickness (c) abaxial epidermal thickness (d) vascular bundle area (e) paranchymatous cell area among diseased and healthy leaf cells of rose

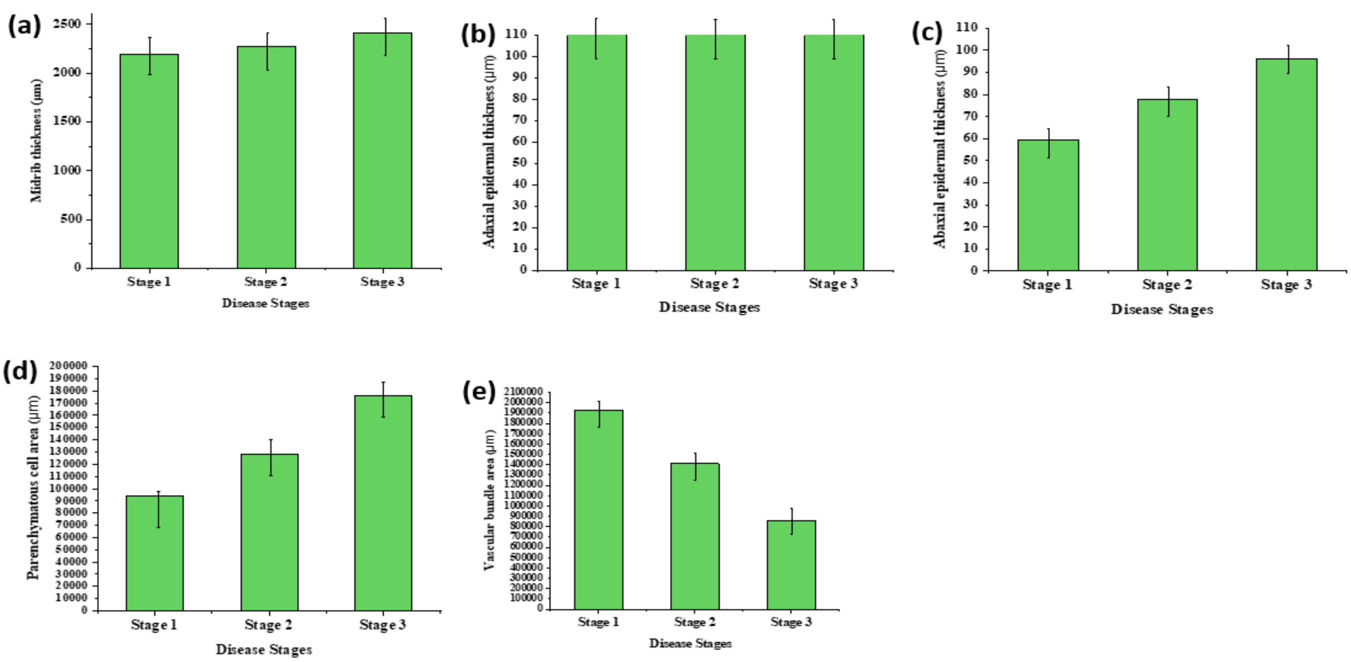

Figure 7. Estimation of (a) midrib thickness (b) adaxial epidermal thickness (c) abaxial epidermal thickness (d) vascular bundle area (e) paranchymatous cell area of diseased cells of rose leaf at different disease stages.

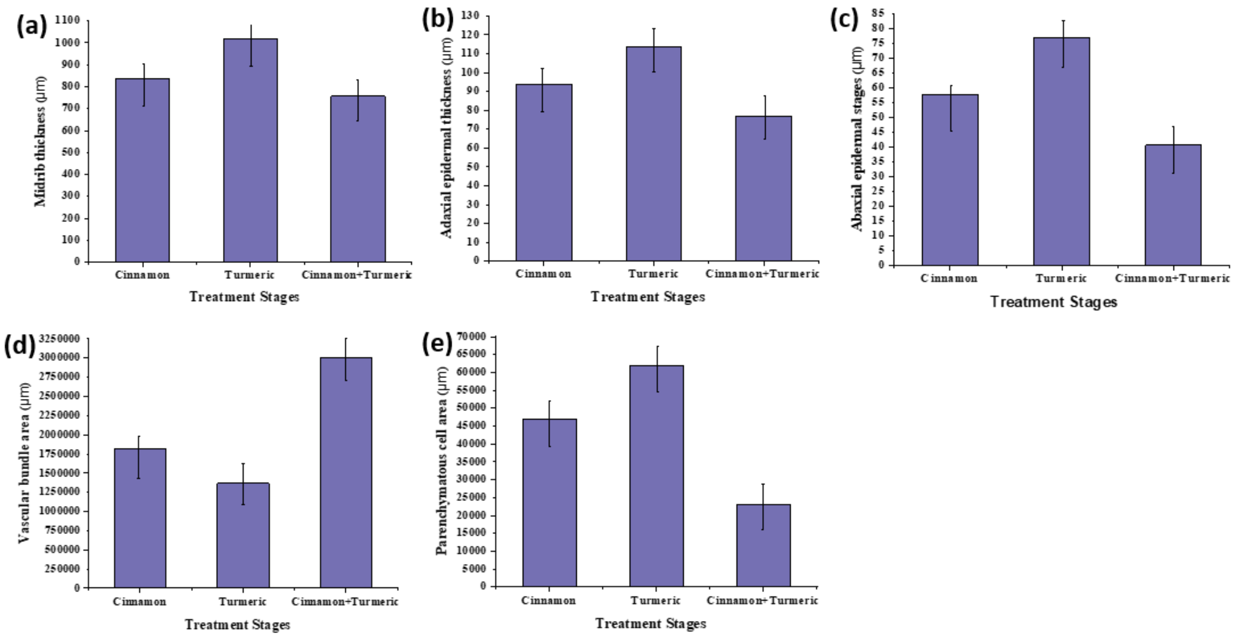

Figure 8. Estimation of (a) midrib thickness (b) adaxial epidermal thickness (c) abaxial epidermal thickness (d) vascular bundle area (e) paranchymatous cell area among rose leaf cells under different treatments.

Principal component Analysis

Principal component analysis (PCA) shows the association among healthy, diseased and phyto-extract treated leaves. Adaxial epidermal thickness (ADET) and abaxial epidermal thickness (ABET) both were strongly linked to the diseased (DS) and turmeric (TR) treated leaves. Midrib thickness (MIT) and parenchymatous cell area (PCA) have a close association with inoculation stage 2 (S2) and stage 3 (S3). Vascular bundle area (VBA) has a direct relation with healthy leaves (HT) and inoculation stage 1 (S1). While there was no association between Cinnamon treated (CN) and Cinnamon + Turmeric treated leaves (C+T) (Figure 9).

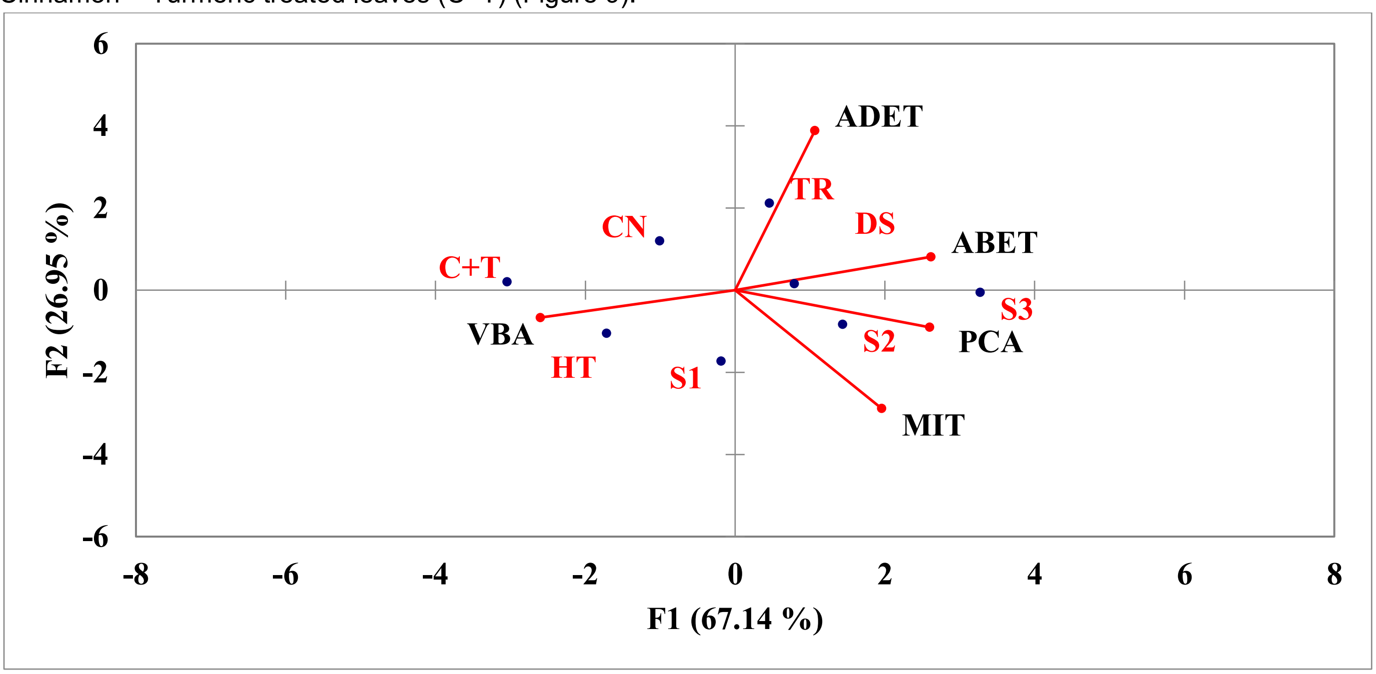

Figure 9. Principle component analysis for understanding of association of all parameters with relation to different aspects of investigation. While, leaf anatomical traits are ADET-Adaxial epidermal thickness, ABET-Abaxial epidermal thickness, PCA-Parenchymatous cell area, MIT-Midrib thickness, VBA-Vascular bundle area, HT-Healthy leaves, DS-diseased leaves, S1-Inoculation stage 1, S2-Inoculation stage 2, S3-Inoculation stage 3, CN-Cinnamon treated leaves, TR-Turmeric treated leaves, C+T-Cinnamon + Turmeric treated leaves.

DISCUSSION

Generally, the fungal plant diseases are being controlled by using synthetic chemical pesticides, which are highly dangerous to animal and human health. So, the scientific community is focusing on biological control strategies to minimize the use of hazardous synthetic chemicals, in that sense different plant extracts, bio fungicides and agro nanotechnology are being highly recommended (El-Baky et al. 2021; Asthana et al. 2001), due to their eco-friendly and frequent biodegradable nature (Sitara et al. 2008). The current study also contained five phytoextracts Cinnamon (*Cinnamomum verum*) Turmeric (*Curcuma longa*) Black pepper (*Piper nigrum*) Ginger (*Zingiber officinale*) and Aloe vera (*Aloe vera*) to assess their antifungal activity against black spot disease of rose under *in-vitro* conditions. Recent investigations disclosed that, Cinnamon demonstrated the most effective results against *Diplocarpon rosae* as compared to other phyto-extracts. Cinnamon contains a number of polyphenolic compounds and cinnamaldehyde that inhibits fungal growth including molds and yeasts (Hamidpour et al. 2015). Our outcomes are also in line with the studies conducted by Sabarinathan et al. (2019), they investigated the antifungal activity of nine different types of phytoextracts against *Diplocarpon rosae* and concluded that among all, the extract of *Zingiber officinale* exhibited significant fungal inhibition (41.85%) as compared to others. Marzani et al. (2021) also determined the antifungal potential of thirteen different phyto-extracts against powdery mildew disease of rose and observed that, the extracts of Ginger and Garlic showed the effective results among respectively.

In current study, cinnamon and turmeric were the most effective treatments under *in-vitro* environments, that’s why these extracts were further investigated as solo and in combination applications against black sport of rose under *in-vivo* conditions. Where, findings indicated that, the minimum disease incidence percentage was recorded by Cinnamon + Turmeric combination as compared to Cinnamon and Turmeric alone. Outcomes of our study is supported by Jeliazkova et al. (2012) who conducted an *in-vivo* experiment for the evaluation of various plant extracts against *Diplocarpon rosae* and found that, the extracts of Scoth spearmint and English thyme showed effective disease control with minimum disease incidence.

Our study also contained anatomical analysis of rose leaves to observe certain alterations in healthy and diseased rose leaf cells. Our findings declared that, in a healthy leaf, both adaxial and abaxial epidermal cells, Parenchyma cells, and vascular bundles were normal in size. Midrib thickness was also found to be normal. While in a diseased leaf, due to fungal penetration, parenchyma cells became larger in size, adaxial epidermis was ruptured, vascular bundle area was increased and vascular bundle area was decreased. These findings were supported by the investigations of Palmer et al. (1987) who performed an experiment in which microscopic examinations revealed the presence of many tissues in a healthy and fully grown rose leaf, including upper and lower epidermal cells, palisade and spongy parenchyma cells having different types of vascular tissues that were surrounded by xylem parenchyma cells and a number of air spaces. While in a diseased rose leaf, upon conidial germination a germ tube is formed which penetrates the cuticle, epidermal cells, palisade and spongy parenchyma cells and xylem parenchyma cells through intercellular, intracellular subcuticular and intramural hyphae and produced the feeding fungal organs called haustoria in these cells. To observe the mode of action and development stages of pathogen *Diplocarpon rosae*, anatomical analysis were conducted at different stages of inoculation. The findings suggested that black spots were started to emerge within a week of inoculation. After microscopic examinations, it was observed that, in the first week, only the adaxial epidermis was found to be ruptured without any effect on other cells. In the second week, fungus was start penetrating into mesophyll cells and in the third week, after complete maturation of spots it was observed that, mesophyll cells become larger than their normal size and vascular bundle area was also increased. Results of our study are in line with the work of Gachomo et al. (2006), where they reported the mechanism of fungal penetration and the formation of various fungal structures into the host tissues. Mechanism of fungal penetration starts with the formation of germ tube upon conidial germination having an appressorium at end. The haustoria were start forming in epidermal cells and later in mesophyll cells through intercellular hyphae. Intramural and subcuticular hyphae give rise to formation of haustoria in epidermal cells as compared to palisade mesophyll cells during disease development (Gachomo and Kotchoni 2007).

CONCLUSIONS

Black spot disease is a potential threat to rose production. This study contained analytical examination to understand the mechanism of pathogenic invasion and its diverse effects on cellular level. The obtained results also showed that the use of phyto-extracts have promising potential against *Diplocarpon rosae.* Further studies are needed to gain more comprehensive insights regarding the active ingredients of phyto-extracts and their mechanism of action against pathogens.

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AUTHOR CONTRIBUTIONS

All the authors contributed equally to this research.

COMPETING OF INTEREST

No conflicts of interest have been disclosed by the authors.

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