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Research Article

Control potency of Trans-cinnamic acid and antifungal metabolites of *Xenorhabdus szentirmaii* against *Alternaria brassicicola*

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ABSTRACT

The, antifungal activities of trans-cinamic acid (TCA) and cell-free supernatant (CFS) of *X. szentirmaii* were evaluated against *Alternaria brassicicola* that cause Black spot disease. The results showed that TCA was more suppressive than CFS of *X. szentirmaii* in the control of *A. brassicicola*. In petri assays, the highest suppressive effect on spore germination was in TCA treatments compared to CFS of *X. szentirmaii*. Moreover, no germ tube elongation was observed in any of tested concentrations (0.25%, 0.50%, 1%, 2%) of TCA. The highest level of mycelial growth inhibitions (100% and 92%) were exhibited by TCA (2%) and TCA (1%), respectively. The curative and protective activity of TCA on disease severity of *A. brassicicola* were also evaluated on broccoli plants. In the curative activity assays, disease severities were 71.00%, 56.33% and 54.03% for control, TCA (1%) and TCA (2%), respectively. Whereas in the protective activity, TCA (2%) suppressed the disease severity significantly greater than TCA (1%). The disease severity of *A. brassicicola* after TCA application on broccoli plants was 25.21% and 17.37% for TCA (1%) and TCA (2%) respectively. Similar data were obtained in the efficacy of TCA on *A. brassicicola*. In the curative activity assays, TCA (1%) and TCA (2%) exhibited the efficacy with rates of 20.57% and 23.02%, respectively. However, TCA (2%) showed significantly higher efficacy than TCA (1%) in the protective activity. The current study provides that TCA has a great potential to suppress *A. brassicicola* and can be a good alternative to synthetic fungicides.

Keywords: *Alternaria, Trans-cinnamic acid, Xenorhabdus*

Alternaria brassicicola'ya karşı Trans-cinnamic Asit ve Xenorhabdus szentirmaii'nin Antifungal Metabolitlerinin Kullanım Potansiyellerinin Araştırılması

ÖZET

Bu çalışmada trans-cinnamik (TCA) ve *Xeorhabdus szentirmaii* bakteri süpernatatının (CFS) kara leke hastalığı etmeni *Alternaria brassicicola*'ya karşı antifungal etkinliği test edilmiştir. Sonuçlara bakıldığında TCA, *X szentirmaii*'nin süpernatantına göre *A. brassicicola* üzerinde daha etkili olmuştur. Petri deneylerinde *A. brassicicola*'nın sporlarının çimlenmesini en fazla baskılayan deney grubunun TCA olduğu görülmüştür. Ayrıca TCA'nın tüm konsantrasyonlarının (%0.25, %0.50, %1, %2) hiçbirisinde çim borusu gelişimini gözlenmemiştir. *A. brassicicola*'nın misel gelişimini en fazla inhibe eden (%100 ve %92 oranlarında) TCA konsantrasyonları ise sırasıyla yüzde 2 ve yüzde 1'dir. *A. brassicicola*'nın hastalık şiddeti üzerine TCA'nın koruyucu ve tedavi edici etkisi brokoli fideleri kullanılarak test edilmiştir. Tedavi edici denemelerde, hastalık şiddeti kotrolde %71 iken TCA (1%) ve TCA (2%) uygulanan brokoli fidelerinde sırasıyla %56.33 ve %54.03 olarak ölçülmüştür. Ancak koruyucu etki denemelerinde TCA(2%), TCA (1%)'e göre hastalık şiddetini daha etkili bir şekilde baskılamıştır. Brokoli fidelerinde gözlemlenen hastalık şiddeti TCA (2%) ve TCA (1%) için sırasıyla %17.37 ve %25.21 olarak ölçülmüştür. TCA'nın *A. brassicicola*'ya etkinlik denemelerinde ise TCA (1%) ve TCA (2%) arasında tedavi

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edicilik bakımından fark görülmemiştir. Yüzde etki değerleri TCA (1%) ve TCA (2%) için sırasıyla %20.57 ve %23.02 olarak hesaplanmıştır. Bununla beraber TCA (2%), TCA (1%)'e göre koruyucu etki bakımından istatiksel açıdan daha yüksek bir etkinlik sergilemiştir. Bu çalışma TCA'nın *A. brassicicola*'ya mükemmel bir kullanım potansiyeli olduğunu ortaya koymaktadır ve sentetik fungusitlere iyi bir alternatif olabileceğini göstermektedir.

Anahtar Kelimeler: *Alternaria, Trans-cinnamic acid, Xenorhabdus*

I. INTRODUCTION

The causing agent of Black Spot Disease, *Alternaria brassicicola*, is an important plant pathogenic fungus which can affectvarious plants including spinach [1], radish [2], cauliflower, celery cabbage, broccoli, [2], [3] and Chinese kale [4], [5]. The pathogen attacks plant's leaves and stems causing brown spots on leaves which can be yellow halos at an early stage. [2], [6]. The pathogen can damp off the seedlings [2]. The spores can penetrate to seed coat and colonize, making the infected seeds the main source for spreading the disease [2], [7]. The most preferred control method of this pathogen is to use chemicalusing fungicide (Iprodione). However, the repeated chemical fungicide applications can negatively affect the consumers, farmers, other non-target organisms, environment and can also cause resistant strains to develop [4], [8].

The bacteria *Photorhabdus* spp. and *Xenorhabdus* spp. have a symbiotic association with entomopathogenic nematodes (EPNs) *Heterorhabditis* and *Steinernema*, respectively [9]. EPNs are lethal insect pathogens that are used to biologically control of insect pests.. In nature, the infective juveniles (IJs) live in soil and carry the bacteria in their intestine. The IJs penetrate the insect hemocoel via mouth, anus or spiracle, or through the cuticle and release the symbiotic bacteria. Subsequently, the bacteria produce and secrete a variety of metabolites, nematodes and bacteria both kill the host within 48-72 h. The metabolites also inhibit the growth of other competitor microorganisms. The nematode and bacterial development are occured until the food sources deplete. Finally, new generation IJs leave the cadaver in 7-15 days to find a new host [10].

Using biological control options (biofungicides etc.) instead of chemical pesticides is an uprising trend in many parts of the world. Thus, discovery of new biological control agents has become a necessity. In the previous studies, a whole range of antimicrobial or antifungal metabolites have been isolated and identified from *Photorhabdus* and *Xenorhabdus* bacteria [9], [11], [12], [13], [14], [15], [16], [17]. Among these compounds, trans-cinnamic acid (TCA) from *Photorhabdus luminescens* [11] and fabclavine from *Xenorhabdus budapestensis* and *X. szentirmaii* [14] have been reported as potential antimycotic control agents. TCA is also a natural product obtained mostly from cinnamon plants.

The suppressive effect of these compounds against a variety of important plant pathogenic fungi have been shown in different studies[18], [19], [20], [21], [22]. Thus, our objective was to determine the suppressive abilities of trans-cinamic acid and cell-free supernatant (CFS) of *X. szentirmaii* against *Alternaria brassicicola* within in vitro and in vivo conditions.

II. MATERIALS AND METHODS

A. I. Phytopathogen culture of *Alternaria brassicicola*

The isolate of *A. brassicicola* was obtained from infected cauliflower leaves collected from Düzce, Türkiye. The fungi were cultured on potato dextrose agar (PDA) in Petri dishes at 27 ℃. Pathogenicity of the isolate was confirmed according to Koch's postulates. The isolate was stored at the Laboratory of Plant Protection, Department of Plant Pathology, Duzce University.

A. 2. Preparation of trans-cinnamic acid solution and cell-free supernatant of *X. szentirmaii*

Trans-cinnamic acid is a natural product obtained mostly from cinnamon plants. The commercial TCA product (Sigma, Germany) was used in this study. To prepare the stock solution, the protocol suggesting by Adlığ and Gülcü [22] was followed. According to protocol, 4.5 g of TCA (≥ 99% purity) was dissolved in 100 mL ethanol (96%).
The bacteria, *Xenorhabdus szentirmaii* DSMZ 16338, was provided from Dr. Hazir's Lab. from Department of Biology in Adnan Menderes University, Aydın, Türkiye. The stock culture was prepared as described by Hazir et al. [21] and stored at -80°C until required. The obtain cell-free supernatant (CFS) including secondary metabolites, the protocol was followed according to Hazir et al. [21] and Shapiro-Ilan et al. [23]. We filtered the CFS with using 0.22 µm millipore filter (Thermo scientific, NY) and stocked into the 50 ml sterile centrifuge tubes at 4℃. The CFS were stored up to two weeks for the experiments.

A. 3. Efficacy of bacterial secondary metabolites and TCA on spore germination of *A. brassicicola*

The inhibitory effect of CFS and TCA on spore germination were evaluated in petri plates. Different concentrations (v / v) of CFS (0, 5, 10 and 15%) and TCA (0, 0.25, 0.5, 1 and 2) were inserted into PDA. To prepare the concentrations, the protocol from Hazir et al. [21] was followed. A 50µl conidia suspension (10^5 spores mL^{-1}) was poured onto PDA in the treatments. The first 100 conidia were counted under the microscope (400×) to determine percentage spore germination [21]. Additionally, the germ tube elongation of germinated spores were measured using a computer software (cell Sens Standard 1.11).

A. 4. Efficacy of bacterial secondary metabolites and TCA on mycelial growth of *A. brassicicola*

The antifungal activity of CFS and TCA were evaluated in petri assays. To evaluate mycelial growth, the method from Hazir et al. [21] was followed. Different concentrations (v/v) of CFS (5, 10 and 15%) and TCA (0.25, 0.5, 1 and 2) were evaluated in the study. A mycelia plug (5 mm diameter) of *A. brassicicola* was placed on the center of petri arena that containing CFS or TCA. Petri dishes were incubated at 25℃ and the diameter of colonies (cm) were measured after five days. There were ten replicates for each treatment. The experiment conducted once.

A. 5. Efficacy of curative and protective activities of TCA against *A. brassicicola*

Based on the data from vegetative growth experiments, TCA (1%) and TCA (2%) were tested for curative and protective activities. Seven–week old broccoli plants were used in the study. The plants were provided from Dikmen Fide Company, Bilecik, Türkiye.

In the protective activity assays, TCA applications were made on broccoli plants firstly. Three mililiter TCA per pot was sprayed on both sides of the leaves. After 24 h, 3 mLof *A. brassicicola* spore suspension (10^6 spores mL^{-1}) were inoculated on the plants.
For curative activity assays, 3 mL spore suspension (10^6 spores mL^{-1}) of *A. brassicicola* were applied on each plant firstly. The plants were incubated for 24 h under moist conditions. Then, TCA were applied on the infected plants. Three mililiter TCA was applied per pot for each concentration. In positive control, the plants were treated with distilled water, while the plants were treated with a spore suspension of the pathogen alone in negative control. Each treatment had twelve replicates and experiment was repeated ones. The plants were placed in a greenhouse in a randomized complete block design. They were incubated at 25 ±3°C and watered daily. The disease severity was the pathogen inoculation as a percentage of lesion area over the total leaf surface. The disease severity was classified into six levels: 0 = no lesion area; 1 = lesion area 1– 20%; 2 = lesion area 21–40%; 3 = lesion area 41–60%; 4 = lesion area 61–80%; 5 = lesion area > 80% [24]. The data for disease severity was recorded after 5 days.

A. 6. Data analysis

The data was analyzed with IBM SPPS, Statistics 22. Data are shown as the mean ± SE. The comparisons between groups were evaluated using analysis of variance (ANOVA) with Tukey's post hoc test. The Abbott formula was used to convert the mean of vegetative growth in petri arena to percentage of the mycelial growth inhibition as well as efficacy (%) of CFS and TCA. Then, the comparison was performed with student-t test. Tawnsend-Heuberger formula was performed to determine disease severity depending on scale values that obtained from pot experiment. Differences at $p < 0.05$ was accepted as statistically significant.

III. RESULTS and DISCUSSION

The TCA was the most suppressive on spore germination of *A. brassicicola*. Additionally, there was a significant difference between germ tube elongation in TCA treatments and *X. szentirmaii* treatments. The percentage spore germination of *A. brassicicola* was the least in the TCA treatments, however fungal spore germination ranged between 95.49 and 98.06 for the control and for all the treatments with *X. szentirmaii* ($F = 1546.904$; $df = 7$; $p < 0.05$). The average germ tube elongation ranged between 73 µm and 138µm for all treatments with *X. szentirmaii* and control ($F = 287.161$; $df = 7$; $p < 0.05$). Whereas no germ tube development was observed in TCA treatments (Table 1).

Table 1. Effects of *Xenorhabdus szentirmaii* and trans-cinnamic acid on germ tube elongation and spore germination

	Control	Xz (5%)	Xz (10%)	Xz (15%)	TCA (0.25%)	TCA (0.5%)	TCA (1%)	TCA (2%)
Germ tube elongation (µm)	108(c)*	138(d)	113(c)	73(b)	0(a)	0(a)	0(a)	0(a)
The inhibition of spore germination (%)	2.23(b)	4.51(b)	1.94(b)	2.78(b)	99(a)	99(a)	100(a)	100(a)

*Different letters in same rows indicate statistical significance ($p<0.05$). Xz: *Xenorhabdus szentirmaii*, TCA: Trans-cinnamic acid.

The results indicate that the highest level of mycelial growth inhibition was exhibited by TCA (2%) followed by TCA (1%) (Figure 1). Additionally, there is a significant difference between

TCA (2%) and TCA (1%). The mycelial growth inhibition rates were 99.53% for TCA (2%) and 91.63% for TCA (1%) on *A. brassicicola*. The mycelial growth inhibition for *X. szentirmaii* supernatant treatments and the rest of the TCAs ranged between 13.19% and 51.48% ($F = 127.6664$; $df = 6$; $p<0,05$).

We evaluated the curative and protective activity of TCA on disease severity of black spot. In the curative activity on disease severity on broccoli plants was 56.33% and 54.03% for TCA (1%) and TCA (2%), respectively. There was no significant difference in the efficacy of curative activity between TCA (1%) and TCA (2%) ($F= 24.1482$; $df= 2$; $p<0.05$) (Figure 2a). The disease severity of *A. brassicola* after TCA application on broccoli plants was 25.21% and 17.37% for TCA (1%) and TCA (2%), respectively. The difference between TCA (1%) and TCA (2%) was statistically significant ($F = 33.2863$; $df = 2$; $p<0.05$) for protective activity on disease severity (Figure 2b).

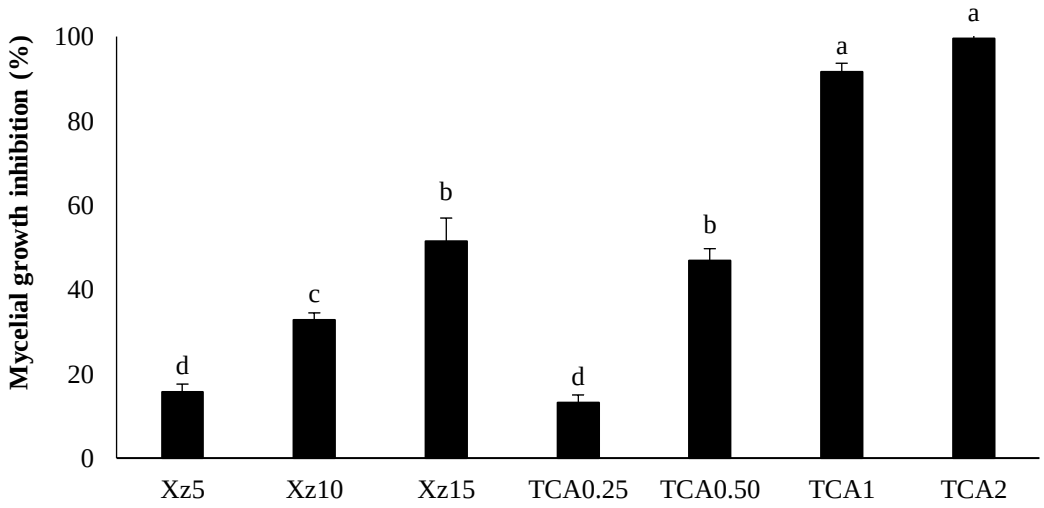

Figure 1. Mycelial growth inhibition (%) of TCA and Xz on *Alternaria brassicicola* on Potato dextrose agar media. Different letters above bars indicate statistical significance ($p<0.05$). Xz: *Xenorhabdus szentirmaii*, TCA: Trans-cinnamic acid.

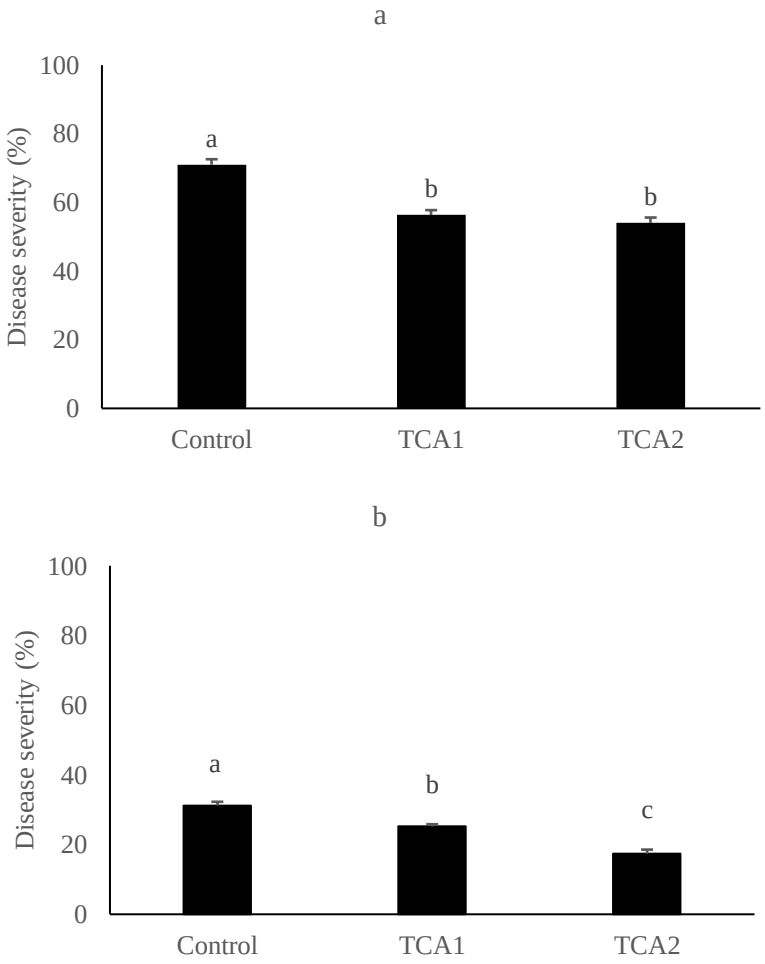

Figure 2. The curative activity (a) and protective activity (b) of trans-cinnamic acid on disease severity of *Alternaria brassicicola*. Different letters above bars indicate statistical significance (Student's t test, $p<0.05$). TCA: Trans-cinnamic acid.

For the curative activity, TCA (1%) and TCA (2%) exhibited the efficacy with a rate of 20,57% and 23,02% respectively. Additionally, no significant difference was found between TCA (1%) and TCA (2%) ($F=0.5762$; $df=1$; $P=0.4637$) (Figure 3a). The protective activity results indicate that TCA (2%) showed higher efficacy than TCA (1%) and the results were statistically significant(1%), $F= 23.3403$, $df=1$, $p<0.05$. The rates of efficacy were 43.88 % and 22.63 % for TCA (2%) and TCA (1%) respectively (Figure 3b).

Previously, different microorganism such as antagonistic fungi [24], endophytic *Streptomyces* bacteria [25], [26] or their crude extracts such as marine algae extracts [27], and medicinal plants extracts [4] were tested to control *A. brassicicola*. This study is the first to evaluate the antifungal activity of *X. szentirmaii* CFS and TCA against *A. brassicicola*.

The results of this study showed that TCA displayed a stronger suppression on the germination of spore and vegetative growth of *A. brassicicola* compared to CFS of *X. szentirmaii*. The cinnamic acid is the natural product of Cinnamomum plants, moreover it has recently been reported from *Photorhabdus luminescens* by [11]. Its significant antifungal activity has been reported by [11], [21], [22], [30], [31], [32], against various plant pathogenic fungi. [32] explained the mode of action of cinnamic acid on *Sclerotinia sclerotiorum*. They indicated that cinnamic acid inhibited the oxalic acid metabolism of *S. sclerotiorum* which is important for pathogenicity and sclerotia development. [33] mentioned that cutinases, lipases, cell wall-degrading enzymes (CWDEs), and proteases are important for the pathogenecity of *A. brassicicola*. TCA might be inhibiting the mechanism of these genes that related to the pathogenicity of *A. brassicicola*. Whereas, our hypothesis has not to be tested yet.

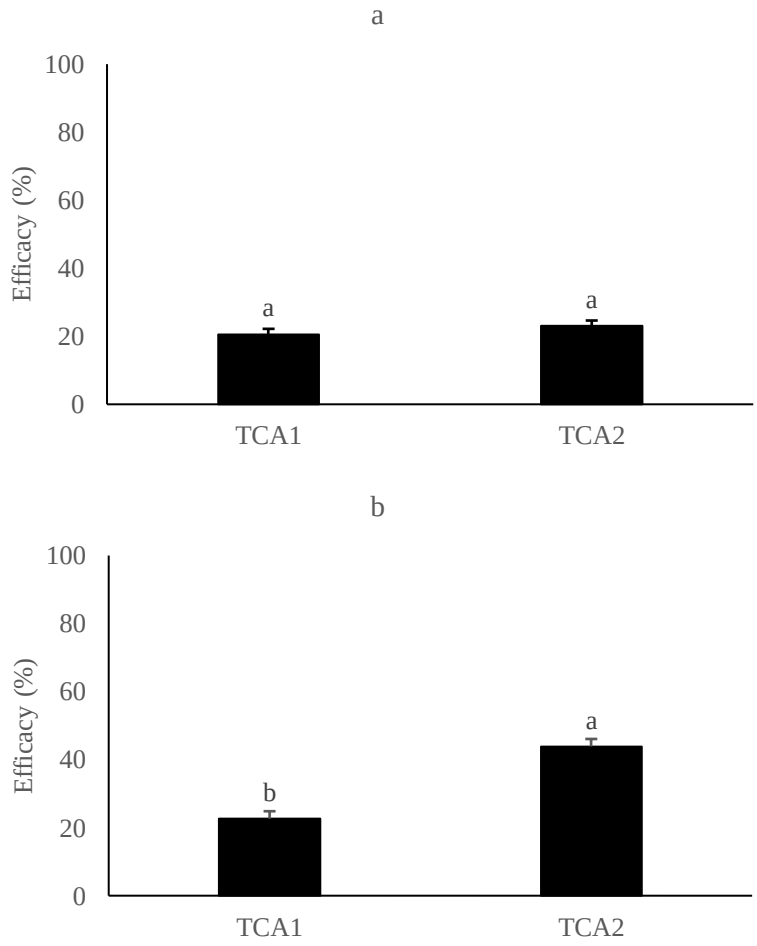

Figure 3. The curative activity (a) and protective activity (b) of trans-cinnamic acid on *Alternaria brassicicola*. Different letters above bars indicate statistical significance (Student's t test, $p<0.05$)

Another point is that either of the tested concentrations of TCA did not exhibit any phytotoxic effect on the broccoli plants in the protective activity assays. This finding is consistent with the data reported by [21]. They observed no phytotoxicity of TCA on various plants such as eggplant, pepper, tobacco, tomato, peach and pecan.

The CFS of *X. szentirmaii* could not provide a great suppression compared to TCA on *A. brassicicola*. The findings of the current study do not support the previous reports. The strong antifungal activity of CFS of *Xenorhabdus* have been reported on various plant pathogen [18], [19], [20], [21], [23], [34]. In our study, we tested *X. szentirmaii* DSM16338 strain. [35] and [21] showed the high efficacy of *X. szentirmaii* DSM16338 antibiotics. Moreover, [13], [14], [36], [37] have identified the compounds of *X. szentirmaii*. Among these compounds, fabclavines become prominent with their antifungal properties. [21] suggested that the CFS of *X. szentirmaii* may be as effective as TCA, if the metabolites would be more concentrated. This may explain the poor efficacy in our study since we applied the diluted form of CFS. . [21] also offered two more probability that may be related with our results; the target pathogen and the manner that w applied.

IV. CONCLUSION

The cinnamic acid has been reported to have various pharmacological actions and inhibition activity against some plant pathogenic fungi in the past decades. The current study provides the first data about

suppressive potencial of antifungal metabolites of *X. szentirmaii* against the target pathogen, *A. brassicicola*. These results have exhibited some promising data. Further studies are needed to show the efficacy of TCA in the field conditions. It can be a good option instead of synthetic fungicides and can be used as a biofungicide for black spot disease in the future.

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