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Histological study of leaves symptoms of *Brassica napus* and *Raphanus brassica* infected by *Alternaria brassicae*

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Abstract

Alternaria leaf spot can be a devastating disease as the mechanism by which the fungus *Alternaria brassicae* (Berk.) Sacc. spread in canola is not yet fully understood. It is essential to understand the infection process and mechanisms of resistance by studying biotic and abiotic factors. In the present study, two abiotic elicitors-Salicylic acid (200 ppm), and Milor MZ (Mancozeb 64% + Metalaxyl 8% WP) fungicide (100 ppm) along with the biotic elicitor *Pseudomonas fluorescens* (PF83), were examined to induce systemic resistance in *Brassica napus* AACC (2n=38) and *Raphanus brassica* AARR (2n=38) against *Alternaria* leaf spot disease. The histological study revealed that *R. brassica* exhibited resistance to *Alternaria* leaf spot disease. Fungal colonies were observed on *R. brassica* leaves at 120 hours post-inoculation (hpi), and Similar observations were seen on *B. napus* leaves after 24 hpi. However, signs of the pathogen, including hyphae, conidia, appresoria, and dead cells, were present on the surface of the infected leaf in both genotypes but were not observed in plants treated with biotic and abiotic elicitors. Thus, the biotic elicitors PF83 and the abiotic elicitors SA and Milor MZ significantly reduced ($P \leq 0.5$) lesion sizes of pathogen at 24, 84, and 120 hpi in both genotypes. Finally, the results suggested that *R. brassica* produced resistance to *Alternaria* leaf spot. Additionally, the application of PF83 isolate is considered a useful tool for enhancing protection methods for canola genotypes.

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Introduction

The Brassicaceae family is economically and industrially significant, including many species important for oil production. *Brassica napus* is one of the top three most valuable oilseed crops, serving as a source of nutrition for humans, forage for animal feed, and raw material for biofuels and soil conditioners. It also holds significant socioeconomic importance across various industries (Borges *et al.*, 2023).

Alternaria brassica is a plant pathogenic fungus and the causal agent of *Alternaria* leaf spot disease. This disease can severely impact crops in the Brassicaceae family, including rapeseed (*B. napus*) and mustard (*B. juncea*), causing significant economic losses. In some cases, such as in Lithuania, it has caused 100% losses of seed yield (Brazauskiene and Petraitiene, 2006), while in Canada, losses have reached up to 42% (Degenhardt *et al.*, 1974).

In general, *A. brassicae* infests the phyllosphere, causing early foliar deterioration, defoliation, and a reduction in the photosynthetic area (Sharma *et al.,* 2002). Moreover, it reduces seed size, yield, and color, leading to a decrease in oil content (Kaushik *et al.*, 1984). The hyphal growth of *A. brassicola* within the leaf tissues of *B. oleracea* results in the loss of host cell integrity and organelle disintegration at different levels (Macioszek *et al.*, 2020). While many methods are available to control *Alternaria* leaf spot disease, they are not always effective (Meena *et al.*, 2010). One of these methods involves using resistance-inducing factors such as Elicitor factors.

Elicitor factors that induce systemic resistance in plants are classified as biotic or abiotic, and as physical or chemical, depending on their origin and molecular structure. External elicitors can be defined as factors that influence the cellular system to build certain compounds involved in defense mechanisms against plant pathogens (Riseh and Vazvani, 2024). Bacteria that colonize plant roots and promote growth are referred to as plant growth-promoting rhizobacteria (PGPR). PGPR influences plant growth in two different ways: directly or indirectly. Non-pathogenic *Pseudomonas* species, a well-known genus for PGPR, are recognized for their antagonistic effects and their impact to stimulate induced systemic resistance (ISR) in plants. This enhances the effectiveness of bio-control strategies, contributing to improved cropping systems (Yu *et al.*, 2022).

To identify the genome (AA, CC or RR) containing the resistance trait against *Alternaria* leaf spot disease, the researcher studied two genotypes: *B. napus* (AACC, 2n=36) and *B. rapa*/*R. sativus* (AARR, 2n = 38). Additionally, a comparative study, between biotic and abiotic elicitors, was conducted to induce systemic resistance in genotypes tested against *Alternaria* leaf spot.

Materials and methods

Plant, pathogen and elicitors materials

Experiments, to evaluate the efficacy of *P. fluorescens* PF 83, salicylic acid, and Milor MZ fungicide (Mancozeb 64% + Metalaxyl 8% WP) as systemic resistance inducers to *Alternaria* leaf spot disease in two spring-type genotypes, *B. napus* (AACC) and *B. raphanus* (AARR), were conducted at the greenhouse of the College of Agriculture, Wasit University, Wasit, Iraq, from October 2022 to May 2023. Whereas Canola seeds, *A. brassica*, *P. fluorescens* PF 83, salicylic acid and Milor MZ fungicide (Mancozeb 64% + Metalaxyl 8% WP) were obtained from the Plant Protection Department, College of Agriculture, Wasit University, to be used in the study.

The *A. brassicae* fungus was maintained and cultured on PDA medium (200 g peeled potato, 20 g dextrose, 15 g agar, and 1 liter distilled water) in the dark at 20 ± 2°C. After 4 days, 5-mm-diameter mycelia agar plugs were punched from the growing margin. For leaf inoculation, ascospores were harvested in sterile distilled water using a sterile brush, and then filtered through four layers of cheesecloth to remove the mycelia. The resulting spore suspension was adjusted to 1×10^4 spores/ml. For *P. fluorescens* was grown in King's broth medium and incubated at 30 ± 1°C for 48 h (King *et al.*, 1954).

Plant cultivation under greenhouse conditions

Experiments were set in appropriate conditions in the greenhouse using mixture of peat moss and sand at a ratio of 1:1 that had been autoclaved twice for 1 h with a 24 h interval and then were placed in the earth pots (25-cm-diamter). 2 cm holes were also made in the bottom of each pot to minimize the loss of excess water. Ten seeds were sown separately in each pot and grown at 20 to 25°C with a cycle of 12 h/12 h light/dark for 35 to 45 days. The irrigation was applied by drenching twice a week. After germination, only five plants were allowed to grow in each pot.

Screening of elicitors based on ISR-eliciting potential and effects on plant growth

For screening the biotic and abiotic elicitors capable of eliciting ISR on thirty days old oilseed plant roots of genotypes were treated with suspensions separately by 100 ml / pot (*P. fluorescens* suspension, salicylic acid (200 ppm) and Milor MZ (100 ppm) by mixing the upper soil surface of each pot in the greenhouse experiments. After 24 hours of treatment, the leaves and stems of plants were inoculated by suspension of *A. brassicae* and separated in 100 ml / pot (reported in 2.1). Each treatment consisted of three replicates.

Histological assessment

Thirty-day-old *B. napus* and *R. brassica* genotypes were treated with elicitors (as reported in 2.3). After one day, the leaves of these plants were inoculated with 0.5 cm plug mycelial of *A. brassicae* placed into the center of each leaf.

The leaves of seedlings were harvested at 24, 72, and 120 hours post-inoculation (hpi) for histological testing. Three samples were collected from each treatment (inoculated and non-inoculated) of both genotypes, with samples taken from separate inoculated pots. Each time interval included samples from three plants. The investigation focused on attack sites on randomly selected leaves using an Olympus microscope. The leaves were stained with Trypan Blue to examine the fungal structures under light microscopy.

Statistical analysis

The experimental design of the greenhouse study was completely randomized, with three replicates for all treatments. The Data were analyzed using analysis of variance with GenStat software, and means ($P < 0.05$) were compared between treatments of oilseed genotypes, biotic and abiotic treatments, and alternria leaf spot disease using least significant difference tests (Gomez and Gomez, 1984).

Results

Effect biotic and abiotic elicitors on the symptoms development upon A. brassicae infection

By testing the effect of pathogen *A. brassicae* on *B. napus* and *R. brassica* genotypes, the results indicated that AARR developed less severe disease symptoms and less tissue damage than AACC (Fig. 1). Soft-rotting necrosis occurred in *B. napus* as early as 1 dpi, in *R. brassica* genotype, necrosis occurred at 2 dpi. The lesion sizes in AACC reached 0.20, 0.85 and 1.55 cm at 24, 48, and 120 hpi, respectively, where they reached 0.00, 0.50 and 1.00 cm respectively in AARR (Fig. 1).

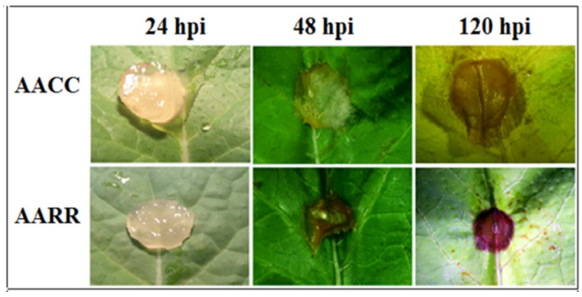

Fig. 1. Disease progression of *A. brassicae* infection on two genotypes leaves of *B. napus* AACC and *R. brassica* (AARR). Leaves of 24, 48, and 120 hours post inoculation (hpi) were inoculated with mycelial discs of *A. brassicae*.

The biotic elicitors PF83 and the abiotic elicitors SA and Milor MZ notably reduced ($P \leq 0.5$) lesion sizes of pathogen at 24, 84, and 120 hpi. In *B. napus* AACC treated with PF83 measured 0.00, 0.43, and 0.75 cm, respectively; with SA, they measured 0.00, 0.50, and 0.77 cm, and with Milor MZ fungicide, they measured 0.00, 0.55, and 0.80 cm. In *R. brassica* AARR, Lesions treated with PF83 measured 0.00, 0.00, and

0.35 cm; with SA, they measured 0.00, 0.00, and 0.46 cm, and with Milor MZ fungicide, they measured 0.00, 0.00, and 0.58cm (Fig. 2).

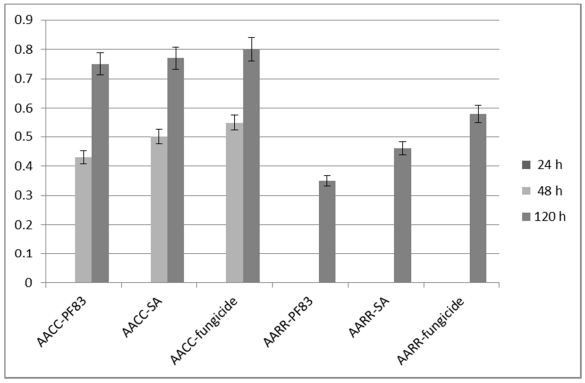

Fig. 2. Leaves of two genotypes: *B. napus* AACC and *R. brassica* (AARR) were inoculated with mycelial agar disc of *A. brassicae*, the lesion size was measured 24, 48 and 120 hours post inoculation (hpi)

Histological study

The germ tube grew into primary hyphae, followed by further hyphal growth. The Fungal hyphae continued to grow on the surface of both genotypes non-treated with biotic and abiotic elicitors, but the growth was lower in *R. brassica* compared to *B. napus*. One or two small lateral branches of hyphae also appeared, but no remarkable difference was observed between the two genotypes treated with elicitors, where the emergence of hyphae was very minimal.

It is observed that there is increased growth in the lateral branches of both genotypes. This growth was clearly visible on the leaf surface of susceptible *B. napus* (AACC). However, branching of the terminal hyphae was also observed in some *R. brassica* (AARR). In stomata, penetration by hyphal apices was found in both the susceptible and resistant host genotypes. These results suggest that both 'AACC' and 'AARR' genotypes are equally susceptible to infection in the early stages (Fig. 3).

At 24 hours post-inoculation (hpi), extensive growth of primary hyphae and lateral branches was seen on the surface of susceptible AACC tissue at certain inoculation sites, appearing as hyphal 'mounds' due to the extensive mycelial growth of A. brassicae. The pathogen's hyphae penetrated the epidermal cells, invaded the palisade mesophyll cells, and grew within intercellular spaces, causing disorganization and browning of the cells beneath the germinated ascospores. This was evident in the darkly stained areas around the dead palisade mesophyll cells in AACC, but no increase in hyphal growth was observed in the resistant AARR (Fig. 3). By 48 hpi, symptoms of infection were visible on the upper surface of B. napus (AACC) leaves, with signs of the pathogen, including hyphae, conidia, appresoria, and dead cells on the infected leaf surface. These Symptoms could be observed with the naked eye and increased over time. AACC showed the pathogen invasion extending to the spongy mesophyll cells, while in AARR, *A. brassicae* was generally confined to the upper epidermis.

At 120 hours post-inculation (hpi), observable signs of the pathogen, including hyphae, conidia, appresoria, dead cells, were present on the infected leaf surface in both genotypes but were absent in both genotypes treated with biotic and abiotic elicitors.

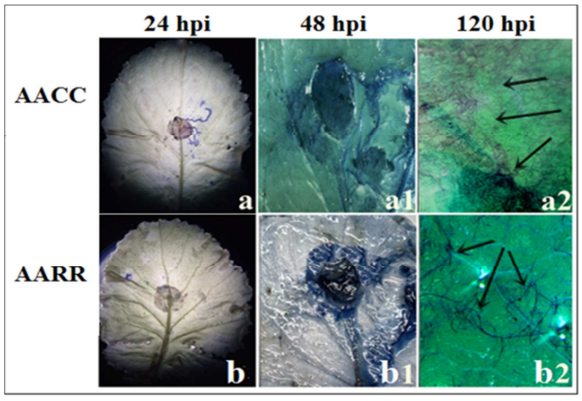

Fig. 3. Symptoms of leaves of *B. napus* (AACC) and *R. brassica* (AARR) infected by *A. brassicae* of 3 time points. Symptom images were (a, b), respectively, and light micrograph were (a1, a2 b1 and b2), respectively. Stocks indicate to colonies and the growth of *A. brassicae* (a2 and b2) and the Black spots mentioned in stocks are dead leaf cells (b2). Scale bars for light micrograph at 120 hpi.

In addition to the hampered hyphae growth on the resistant 'AARR', a significant interaction was observed in AARR at 48 and 120 hpi. In some

instances, the protoplast of the hyphal cells was extruded, which was accompanied by either an increase in hyphal cell diameter and/or cytoplasmic shrinkage. The black spots observed in the samples represent dead leaf cells resulting from hypersensitivity, a type of resistance that demonstrates the viability of the AARR genotype in its rapid response to resistance after treatment with elicitors (Fig. 3).

Dissection

Alternaria brassicicola belongs to the large fungal division ascomycota (Ascomycetes), order Pleosporales. This fungus, which causes *Alternaria* leaf spot in the Brassica family, is one of the most devastating diseases worldwide. *A. brassicicola* is spread by insects- and wind, infecting mature plants and primarily targeting older leaves. At advanced stages of infection, it can lead to the decay of the entire plant (Nowicki *et al.*, 2012). Macioszek *et al.* (2020) reported that infection with the *A. brassica* fungus led to a decrease in chlorophyll content in *B. oleracea* after 48 days of infection.

Systemic resistance using biotic and abiotic elicitors is an effective defense mechanism against phytopathogens. In this study, the results showed the ability of biotic and abiotic elicitors to induce systemic resistance in both tested genotypes. The defense mechanisms triggered by general elicitor's contributed to the plants' innate resistance.

P. fluorescens PF83 and abiotic elicitors played an important role in reducing the growth of the lateral branches and terminal hyphae on both genotypes; hyphal invasion was confined to the upper epidermal layer. At 24 hpi and 48 hpi, hyphal growth was significantly impeded on the surface of resistant 'AARR', suggesting that this genotype produces certain fungistatic or antifungal compounds, as proposed by Blakeman and Sztejnberg (1973) in Beta vulgaris against *Botrytis cinerea* in *Linum usitatissimum* against *Melampsora lini.* The resistance in 'AARR' appears to be due to the hypersensitive reaction (HR) as localized necrosis of the palisade mesophyll cells near the infection site was observed at 120 hpi. This result aligns with previous finding by Garg *et al.* (2008). However, these results indicate that the AARR genotype is resistant to SSR disease, while AACC is non-resistant, suggesting that the RR genotype is responsible for resistance. Al-Lami *et al.* (2023) also pointed out that many genotypes of rapeseed are resistant to *Alternaria* leaf spot disease.

In one of the previous studies, it has shown that bacterial *P. fluorescens* is able to produce some of the materials capable of stimulating the systemic resistance, such as SA, pseudobactin, pseudomonine and a siderophore containing SA (Mercado-Blanco *et al.*, 2001).

Pseudomonas spp. was capable of inducing resistance to *Botrytis cinerea* in grapevine by inducing low levels of phytoalexin in the host cells. Compounds such as SA, pyochelin, DAPG and/or pyoverdin are potentially effective in inducing or priming defense responses in grapevine cells (Verhagen *et al.*, 2010).

These results show that SA and Milor MZ are effective at inducing systemic resistance in plants inoculated and infected with pathogen. Additionally, the resistance levels were increased in both genotypes of plants treated with salicylic acid (SA) and Milor MZ. Salicylic acid (SA) played a crucial role in stimulating systemic resistance to previous pathogen attacks by activating genes associated with resistance. Many studies have highlighted this, including research on peanut against *Alternaria alternata* (Chitra *et al.*, 2008), and on *B. napus* cv. GSC 5 and *B. juncea* cv. ELM 079 against *Alternaria* blight disease caused by *Alternaria brassicae* (Sangha *et al.*, 2007).

The importance of SA lies in its role in the development of systemic acquired resistance (SAR) in hosts, enabling them to defend against pathogens. The SAR response leads to the rapid accumulation of several pathogenesis-related proteins (PR-Protein). *PR-1, PR-2* and *PR-5* genes, which are markers for SAR dependent systemic on salicylic acid (SA), were

examined in the shoots and roots of tomato plants infected by root-knot nematodes (RKNs).

The results identified that the expression of these genes was up-regulated in plants pre-treated with SA, enhancing resistance pathogen (Molinari *et al*., 2014).

Conclusion

The present study demonstrated that the RR genome is responsible for disease resistance in oilseed *R. brassica* (AARR) against the infection process of *A. brassicae*. It is recommend to use *R. brassica* as resistant genotype for oilseed production, and the application of beneficial biotic and abiotic elicitors for protection and increased oilseed yield is now being practiced in agriculture.

References

Al-lami HFD, **You MP**, **Banga SS**, **Barbetti MJ**. 2023. Novel resistances provide new avenues to manage *Alternaria* leaf spot (*Alternaria brassicae*) in canola (*Brassica napus*), mustard (*B. juncea*), and other Brassicaceae crops. Plant Disease **107**(2), 372–381.

Blakeman JP, **Sztejnberg A**. 1973. Effect of surface wax on inhibition of germination of *Botrytis cinerea* spores on beetroot leaves. Physiological Plant Pathology **3**(2), 269–278.

Borges CE, **Von dos Santos Veloso R**, **da Conceição CA**, **Mendes DS**, **Ramirez-Cabral NYZ**, **Shabani F**, **Shafapourtehrany M**, **Nery MC**, **Siqueira da Silva R**. 2023. Forecasting *Brassica napus* production under climate change with a mechanistic species distribution model. Scientific Reports **13**(12656). https://doi.org/10.1038/s41598-023-38910-3.

Chitra K, **Ragupathi N**, **Dhanalakshmi K**, **Mareeshwari P**, **Indra N**, **Kamalakannan A**, **Sankaralingam A**, **Rabindran R**. 2007. Salicylic acid induced systemic resistance on peanut against *Alternaria alternata*. Archives of Phytopathology and Plant Protection **41**(1), 50–56. https://doi.org/10.1080/03235400600655263.

Degenhardt KJ, **Skoropad WP**, **Kondra ZP**. 1974. Effects of *Alternaria* black spot on yield, oil content, and protein content of rapeseed. Canadian Journal of Plant Science **54**, 795–799.

Garg H, **Sivasithamparam K**, **Banga SS**, **Barbetti MJ**. 2008. Cotyledon assay as a rapid and reliable method of screening for resistance against *Sclerotinia sclerotiorum* in *Brassica napus* genotypes. Australasian Plant Pathology **37**, 106–111.

Gomez KA, **Gomez AA**. 1984. Statistical procedures for agricultural research. John Wiley and Sons, Inc., London, UK (2nd ed.), pp. 13–175.

Kaushik C, **Saharan G**, **Kaushik J**. 1984. Magnitude of loss in yield and management of *Alternaria* blight in rapeseed-mustard. Indian Phytopathology **37**, 398.

King EO, **Word MK**, **Raney DE**. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine **414**, 301–307.

Macioszek VK, **Gapińska M**, **Zmienko A**, **Sobczak M**, **Skoczowski A**, **Oliwa J**, **Kononowicz AK**. 2020. Complexity of *Brassica oleracea–Alternaria brassicicola* susceptible interaction reveals downregulation of photosynthesis at ultrastructural, transcriptional, and physiological levels. Cells **9**(10), 2329. https://doi.org/10.3390/cells9102329.

Meena PD, **Awasthi R**, **Chattopadhyay C**, **Kolte S**, **Kumar A**. 2010. *Alternaria* blight: A chronic disease in rapeseed-mustard. Journal of Oilseed Brassica **1**(1), 1–11.

Mercado-Blanco J, **van der Drift KM**, **Olsson PE**, **Thomas-Oates JE**, **van Loon LC**, **Bakker PA**. 2001. Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. Journal of Bacteriology **183**(6), 1909–1920. https://doi.org/10.1128/JB.183.6.1909-1920.2001.

Molinari S, **Fanelli E**, **Leonetti P**. 2014. Expression of tomato salicylic acid (SA)-responsive pathogenesis-related genes in Mi-1-mediated and SA-induced resistance to root-knot nematodes. Molecular Plant Pathology **15**(3), 255–264.

Nowicki M, **Nowakowska M**, **Niezgoda A**, **Kozik EU**. 2012. *Alternaria* black spot of crucifers: Symptoms, importance of disease, and perspectives of resistance breeding. Vegetable Crop Research Bulletin **76**, 5–19. https://doi.org/10.2478/v10032-012-0001-6.

Riseh RS, **Vazvanim MG**. 2024. Unveiling methods to stimulate plant resistance against pathogens. Frontiers in Bioscience **29**(5), 188. https://doi.org/10.31083/j.fbl2905188.

Sangha MK, **Atwal AK**, **Sandhu PS**, **Bal RS**, **Banga SS**. 2007. Salicylic acid induces resistance to *Alternaria* blight in crop *Brassica* species. Plant Protection: Diseases, 137.

Sharma G, **Kumar VD**, **Haque A**, **Bhat S**, **Prakash S**, **Chopra V**. 2002. *Brassica* coenospecies: A rich reservoir for genetic resistance to leaf spot caused by *Alternaria brassicae*. Euphytica **125**, 411–417. https://doi.org/10.1023/A:1016050631673.

Verhagen BW, **Trotel-Aziz P**, **Couderchet M**, **Höfte M**, **Aziz A**. 2010. *Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is associated with induction and priming of defence responses in grapevine. Journal of Experimental Botany **61**(1), 249–260.

Yu Y, **Gui Y**, **Li Z**, **Jiang C**, **Guo J**, **Niu D**. 2022. Induced systemic resistance for improving plant immunity by beneficial microbes. Plants **11**(3), 386. https://doi.org/10.3390/plants11030386.