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Effect of fungal elicitors on morphophysiological characteristics and resistance to gray mold caused by *Botrytis cinerea* in cut flowers of rose

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Abstract

The present study aimed to enhance the vase life, maintain quality, and reduce the percentage of infection with gray mold caused by *Botrytis cinerea* using fungal elicitors on cut flowers of roses. A factorial experiment was performed on a completely randomized design with three replications. The eight treatments were as follows: three concentrations of fungal elicitor (600, 800 and 1000 ppm), the three concentrations along with *B. cinerea* fungal spores, sterile distilled water (as negative control), and *B. cinerea* spore suspension (positive control). Vase life, cell membrane stability index, superoxide dismutase, catalase, carbohydrate content, and disease severity on the leaves were measured during the experiment. The measurements were carried out on days 3, 7 and 10 after spraying the spores. The results showed that treating rose flowers with the elicitor (1000 ppm) improved soluble carbohydrate preservation, cell membrane stability index, relative water content, catalase enzyme, and leaf superoxide dismutase, resulting in a significant increase in vase life (15.66 days) compared to the control (8.66 days) ($P \leq 0.01$). Evaluating the disease index also showed that the elicitor (1000 ppm) along with *B. cinerea* spores caused a significant increase in vase life (12.33 days) compared to the positive control (7.66 days) ($P \leq 0.01$). The disease severity in treated samples was lower than that of the *B. cinerea* treatment as control.

Key words: Vase life, RWC, CAT, SOD, soluble carbohydrates

Introduction

Rose (*Rosa hybrida*) is recognized as a member of the Rosacea family. Gray mold is one of the most destructive diseases of greenhouse crops, which causes a severe decline in the economic value of ornamental plants (Liu *et al.*, 2021). Gray mold is caused by *Botrytis cinerea* with the sexual form *Botryotinia fuckeliana* haploid, which is filamentous and heterothal. This species of fungus is in the phylum of "true fungi" (Elad *et al.*, 2007). Chemical fungicides are commonly used to control *B. cinerea* in cut flowers in greenhouse conditions in the pre-harvest and postharvest stages (Bui *et al.*, 2019). However, the overuse of chemical fungicides is leaading fungal resistance and public concerns about human health and environmental hazards (Adeniyi *et al.*, 2021). Recently, chemical treatments have been overtly aimed at reducing *B. cinerea* contamination in rose cultivars (Muñoz *et al.*, 2019). Elicitors are compounds of biological or non-biological origin that can induce physiological changes and accumulate phytoalexin, along with the biosynthesis and accumulation of secondary metabolites via defence responses (Saha and Pal, 2021). They also enable plants to resist pathogenic damage (Subban *et al.*, 2019). Methyl jasmonate and salicylic acid elicitors in *Taxus* spp increased paclitaxel yield in several ways (Ahsan *et al.*, 2012). Bioelicitors include polysaccharides, proteins, glycoproteins, and wall fragments of fungi, plants (cellulose and pectin) and microorganisms (*i.e.* chitin and glucan) (Vasconsuelo and Boland, 2007). Fungal elicitors such as *Botrytis cinerea*, *F. oxysporum*, and *Phomaexigua* significantly induce the production of monolignolic compounds in flax (*L. usitatissimum*) cells. In response to fungal elicitors, lignin levels increased significantly in spruce and flax suspension cultures. Reactive oxygen species are highly poised to attack cell membranes, making it reasonable to believe that a decrease in membrane stability is likely due to an enhanced activity of reactive oxygen species and a lowered activity of antioxidant enzymes (Ezhilmathi *et al.*, 2007). The present study aimed to improve the vase life, maintain the quality, and reduce the infection with gray mold caused by *B. cinerea* using fungal elicitors on cut flowers of roses.

Materials and methods

Cut flowers of *Rosa hybrida* ('Samourai' cultivar) were collected early in the morning, for the freshness and water content. The flower branches were cut to a length of 50 cm and then transferred to the horticulture laboratory. In order to reduce the variability among the flower branches and to minimize error, the cut flowers were kept uniform in terms of stem height and number of leaves. During daytime hours, refrigerator doors were opened and closed several times to allow ventilation and prevent the accumulation of ethylene. Fungal elicitors were prepared by processing *B. cinerea*. The fungus was first added to a 250 mL Erlenmeyer flask containing 50 mL of culture medium (including malt extract 3

g/L, yeast extract 3 g/L, peptone 5 g/L and glucose 10 g/L with pH = 6.2). It was then placed on a shaker for six days at a speed of 200 rpm and a temperature of 25 °C with a cycle of 16 hours of light and 8 hours of darkness. The liquid phase of the culture medium was collected by passing through Watman No. 1 filter paper. The resultant solution was centrifuged at 5000 rpm for 15 minutes. The mushroom body was washed several times with distilled water and then dried in an oven at 40 °C before being crushed in a mortar. The dry powders (10 g) were dissolved in 100 mL of distilled water,, pressed at 15 pound per square inch, and autoclaved at a temperature of 121 °C for 20 minutes. The resultant mixture was then centrifuged at 5000 rpm for 10 minutes and the supernatant was collected as a cell extract. This liquid was stored as a fungal elicitor at 4 °C (Baldi *et al.*, 2009). The flowers were placed in solutions containing fungal elicitors at concentrations of 600, 800 and 1000 ppm for 24 hours and then transferred to distilled water until the end of the experiment. Some of the flowers were sprayed with *B. cinerea* spores on the third day after 24 hours of treatment with high concentrations of fungal elicitors and then specific traits were studied to describe pathogenicity. The distilled water was changed daily. The negative control treatment was initially sprayed with distilled water and the positive control was without any treatment, although initially sprayed with *B. cinerea* spores and then placed in distilled water. The treatments were as follows: 1) elicitor 600 ppm, 2) elicitor 600 ppm + treatment with *B. cinerea* fungal spores, 3) elicitor 800 ppm, 4) elicitor 800 ppm + treatment with *B. cinerea* fungal spores, 5) elicitor 1000 ppm, 6) elicitor 1000 ppm + treatment with *B. cinerea* fungal spores, 7) negative control (sterile distilled water), and 8) positive control (*B. cinerea* spore suspension). For inoculum preparation, 10-day cultures of *B. cinerea* were used in vase on dextrose agar (PDA) medium. To prepare the spore suspension, sterile distilled water was applied at a concentration of 80% and added to the fungus culture for 10 days with a volume ratio of one percent. The culture medium was shaken vigorously so that the spores were ultimately suspended. Then, the spores in the suspension were counted and the concentration of spores was maintained at 2×10^5 per mL. This spore suspension was used for performing pathogenic evaluations. The room in which experiments were performed had standard conditions in terms of temperature, light, photoperiod and relative humidity. Relative humidity was read (75%) by a hygrometer. The light intensity was 80-82 lux.

Postharvest life refers to the number of days from harvest to senescence, which ends with symptoms such as the wilting of petals, flower neck flexion and the shedding of petals. Relative leaf water content (RWC) was measured by considering a certain amount of leaves and weighing their fresh weight (FW). The samples were placed in a container containing distilled water for 24 hours in a cool place, and then dried on filter paper. Their weight was recorded again (SW) and, finally, the samples were placed in an oven at 70 °C for 48 hours. After complete drying, their final weight was recorded (DW) and the water content was calculated using the following formula (Beltrano and Ronco, 2008).

RWC = FW–DW / DW–SW × 100

FW: leaf fresh weight, DW: leaf dry weight, SW: leaf saturation weight

Leaf cell membrane stability index was measured by sampling 0.5 g fresh leaves which were crushed and placed in falcons filled with 10 mL of distilled water. The falcons were then placed in a Ben Marie at 30 °C for 1 hour. After removing the samples, the amount of soluble salts was measured using an electrical conductivity meter or EC meter (ECL). The falcons were then placed in an autoclave for 120 minutes at 120 °C. After cooling, the amount of soluble salts was read again (EC2) using a salinity meter (EC meter). Finally, ion leakage was calculated using the following formula (Ezhilmathi *et al.*, 2007).

MSI = 1-(EC1 / EC2) × 100

MSI: Membrane Stability Index, EC1: Electrical Conduction in the first stage, EC2: Electrical Conductivity in the second stage

Superoxide dismutase (SOD) enzyme activity was measured by the inhibition of ability in light reduction aimed at nitroblutetrazolium (NBT) at a wavelength of 560 nm. The activity of this enzyme was investigated by a photometer. The main reaction buffer was 100 mM phosphate buffer with a pH value of 7.5. Then, 100 µM EDTA, 75 µM nitroblutetrazolium, 12 µM methionine, 2 µM riboflavin and Triton X 100 (0.025%) were prepared for the reaction solution. Then, 290 µL was added from the main buffer to each well, and 5 µL of the 2 µM riboflavin buffer was added to the reaction mixture. The device was calibrated at 560 nm. Protein extract (10 µL) was used for measuring each sample. Extraction and measurement of catalase enzyme (CAT) activity was based on the reduction of hydrogen peroxide absorption, using a spectrophotometer at a wavelength of 240 nm for 30 seconds. The reaction mixture consisted of sodium phosphate buffer (20 mM) (pH = 7) and hydrogen peroxide (H_2O_2) (20 µL, 30%) as an electron acceptor. To measure leaf soluble carbohydrates, 0.5 g of leaf sample was taken and 5 mL of ethanol (95%) was added in a Chinese mortar to release sugars. Then, 5 mL of ethanol (70%) was added twice each time and the solution was poured into the test tube. The extract was centrifuged at 3500 rpm for 10 minutes. Then, 0.1 mL of the methanolic extract was separated and 3mL of anthrone (150 mg anthrone + 100 mL sulfuric acid (72%)) was added, and then it was placed in a hot bath (90 °C) for 10 minutes. After cooling of samples, their absorbance was read at 625 nm with a spectrophotometer. Data were analyzed by SAS software and mean values were compared using LSD test ($P \leq 0.05$).

Results and discussion

Vase life: According to the results, the effect of different levels of elicitor was significant on vase life ($P \leq 0.01$). The main effect of treatment on cut flowers indicated that the longest vase life occurred in response to the elicitor 1000 ppm treatment, which had a significant difference with both controls (Table 1). Also, the shortest flower longevity was observed in samples treated with *B. cinerea* as a positive control treatment, significantly different from the negative control. Among the flowers sprayed with *B. cinerea* spores after treatment with elicitors, the concentration of 1000 ppm elicitor had the longest vase life. Elicitors increased rose vase life even after spraying with *B. cinerea*, compared with the control plants.

Leaves are known to age under the influence of physiological age and internal and external factors. Although the aging process is slower in leaves and vegetative tissues than in petals, aging

Table 1. The main and interaction effects of the diffrent treatments on six characters

Treatments	Vase life (d)	Soluble carbohydrates (mg/g FW)			EC (%)		
		3d	7d	10d	3d	7d	10d
Water	8.66 f	0.53cd	0.5d	0.45e	39.74 de	35.5 ef	32.75 ef
Botrytis	7.66 g	-	0.44e	0.4f	-	30.02 f	28.26 f
Elicitor 600	13.66 bc	0.6ab	0.57bc	0.52cd	49.17 ab	47.55 bc	42.88 cd
Elicitor 600+Botrytis	10.33 e	-	0.53cd	0.49d	-	40.33 de	34.24 ef
Elicitor 800	14.66 ab	0.61ab	0.59ab	0.55cd	53.52 ab	48.02 ab	44.57 c
Elicitor 800+Botrytis	11.33 de	-	0.55cd	0.51d	-	42.56 cd	36.86 ef
Elicitor 1000	15.66 a	0.62a	0.6ab	0.57bc	55.76 a	50.81 ab	47.31 bc
Elicitor 1000+Botrytis	12.33 cd	-	0.56bc	0.52cd	-	45.74 bc	37.56 ef
Treatments	RWC (%)	CAT (U/Mg Protein)			SOD (U/Mg Protein)		
		3d	7d	10d	3d	7d	10d
Water	68.73 e	0.027 fj	0.03 fj	0.042 de	0.6 ef	0.63 de	0.67 cd
Botrytis	61.71 f	-	0.026 j	0.032 e	-	0.59 f	0.61 ef
Elicitor 600	82.61 bc	0.037 ef	0.044 cd	0.056 ab	0.65 de	0.7 cd	0.73 bc
Elicitor 600+Botrytis	72.92 de	-	0.037 ef	0.045 cd	-	0.648 de	0.685 cd
Elicitor 800	83.83 ab	0.039 de	0.048 bc	0.058 ab	0.67 cd	0.73 bc	0.75 ab
Elicitor 800+Botrytis	73.83 cd	-	0.039 de	0.047 cd	-	0.658 de	0.698 cd
Elicitor 1000	84.93 a	0.04 de	0.05 bc	0.06 a	0.7 cd	0.75 ab	0.77 a
Elicitor 1000+Botrytis	75.3 cd	-	0.04 de	0.05 bc	-	0.665 de	0.702 bc

in leaves is stimulated by biological and abiotic stresses, such as the removal of roots and low light intensity. It also leads to many metabolic changes in cells, including enhanced activity of proteases, nucleases and chlorophylls which, in turn, reduce the amount of proteins, RNA and chlorophyll (Nasiri *et al.*, 2020). Parnak (2016) reported that the treatment with 500 ppm cell elicitors increased vase life and carbohydrate content of leaves and petals in cut flowers of roses.

A comparison of the mean values regarding treatment effects showed that leaf soluble carbohydrates decreased with time (Table 2). The highest amount of carbohydrates was observed on the third day (elicitor treatment, 1000 ppm), significantly different from the negative control and the lowest amount was observed on the tenth day under the application of *B. cinerea*. On the tenth day, among the flowers sprayed with *B. cinerea* spores, all elicitor concentrations increased the leaf soluble carbohydrates compared to the negative control.

Rose flowers are usually harvested at the bud stage, so large amounts of soluble carbohydrates are needed to make the flowers bloom (Minde, 2019). Since the short life of rose flowers is attributed to vascular occlusion, which restricts the flow of water into the stem, treatment of flowers with cellular elicitors may induce defense enzymes against pathogens and prevent occlusion. The vascular nature of the stem is thought to reduce the resistance of the stem to water flow, increase absorption of the treatment solution, maintain water balance and, finally, increase the vase life of flowers (Sidhdharth and Nivethaa, 2020). Carbohydrates in flowers are needed for respiratory reactions. For cell expansion and growth, osmotic potential is essential for assisting plant cells in absorbing water. Therefore, the accumulation of sugars in cells can increase water absorption (Yamada *et al.*, 2007).

Leaf cell membrane stability index: Based on the results, the effects of treatment, time and their interaction were significant ($P \leq 0.01$) on leaf cell membrane stability index. The results revealed that the leaf cell membrane stability index decreased over time. The elicitor treatments had a positive effect and maintained cell membrane stability better than the negative control. On the tenth day of the treatment, best cell membrane stability was observed in elicitor treatment with a concentration of 1000 ppm, which was significantly different from both negative and positive controls. Also, among the flowers sprayed with *B. cinerea* spores, elicitor 1000 ppm treatment was more effective as compared to two other concentrations. Finally, elicitor 1000 ppm treatment was determined as the most effective treatment in increasing the cell membrane stability index. The aging process develops by increasing ion leakage in the membrane (El-Nabarawy *et al.*, 2018). Naturally, over time, and as the petals age, the membrane's permeability is impaired due to the reduction of membrane proteins. The decrease in proteins is a prelude to ion leakage and reduces the stability of the cell membrane. A strong correlation has been reported between increased membrane leakage and phospholipid degradation in older flower membranes (Ghorbani *et al.*, 2018). Membrane stability index signifies membrane health and stability during postharvest life. The higher value of this index, the better the condition of the cell membrane and the treatments used, which indicate higher membrane stability and have favourable effects on the stability of the cell membrane (Ren *et al.*, 2017). The cell stability decreases over time (after harvest) due to the gradual aging of cut flowers. In general, the aging of flower petals is related to regular biochemical and physiological processes. These processes include hydrolytic enzymatic activities, reduction of macromolecules, increase of respiratory activity and decrease of cell membrane strength, as well as ion leakage and cell disintegration which entail a similar weight loss pattern (Taiz and Zeiger, 2006). Flowers with fungal elicitors probably prevent bacteria growth at the stem's distal end due to reduced electrical conductivity. The overall decrease in electrical conductivity through the vase life occurs due to the expansion and swelling of the pectin in membrane cavities (Lü *et al.*, 2010), whereas, elicitors are likely to reduce this effect.

Relative leaf water content: A comparison of the mean effects of treatments on relative leaf water content showed that treatment with elicitors increased in the RWC and the best concentration was elicitor 1000 ppm which differed significantly with the two other concentrations. Among the flowers sprayed with *B. cinerea* spores on the third day after treatment with elicitors, the 1000 ppm concentration was the best concentration, which improved

Table 2. Comparison of the mean values regarding treatment effects

Treatments	Time (day)	Carbohydrate (Mg/g FW)	SOD (U/Mg Protein)	CAT (U/Mg Protein)	EC (%)
Water	3	0.53	0.6	0.027	39.7
Elicitor 600 ppm		0.6	0.65	0.037	49.17
Elicitor 800 ppm		0.61	0.67	0.039	53.52
Elicitor 1000 ppm		0.62	0.7	0.04	55.76
Water	7	0.5	0.63	0.03	35.5
B.cinerea		0.44	0.59	0.026	30.02
Elicitor 600 ppm		0.57	0.7	0.044	47.55
Elicitor 800 ppm		0.59	0.73	0.048	48.02
Elicitor 1000 ppm		0.6	0.75	0.05	50.81
Elicitor 600ppm+*B.cinerea*		0.53	0.64	0.037	40.33
Elicitor 800ppm+*B.cinerea*		0.54	0.65	0.039	42.56
Elicitor 1000ppm+*B.cinerea*		0.55	0.66	0.04	45.74
Water	10	0.45	0.67	0.042	32.75
B.cinerea		0.4	0.61	0.032	28.26
Elicitor 600 ppm		0.52	0.73	0.056	42.9
Elicitor 800 ppm		0.55	0.75	0.058	44.5
Elicitor 1000 ppm		0.57	0.77	0.06	47.3
Elicitor 600ppm+*B.cinerea*		0.49	0.68	0.045	34.24
Elicitor 800ppm+*B.cinerea*		0.5	0.69	0.047	36.86
Elicitor 1000ppm+*B.cinerea*		0.52	0.7	0.05	37.5
LSD		0.11	0.064	0.014	16.32

the RWC better than the positive control. Water relations are another factor affecting the vase life of cut flowers (Van Doorn, 2012). A decrease in RWC can occur due to the block of water flow in tissues, especially in vascular tissues, a consequence of which is the accumulation of microorganisms (fungi and bacteria) (Van Doorn, 2012). Some studies have shown that using flower preservatives can increase the vase life of cut flowers by maintaining an optimal water balance in the cut flowers, which can be due to increased water absorption or reduced water loss (Shan and Zhao, 2015). Maintaining an optimal water balance in tissues is a fundamental issue for cut flowers. Vascular occlusion may occur due to several factors, including bacteria, air bubbles, and physiological responses to cell death. The water content indicates the turgidity and freshness of the petals of the cut flowers (Kumar *et al.*, 2017). Higher levels of turgidity can make the petals last longer. Vascular occlusion may occur due to the presence of bacteria, thereby reducing water absorption and causing stem deformation as well as petal wilting in cut flowers. Therefore, water balance and turgidity are important factors in extending vase life (Solgi *et al.*, 2009). Similar to our results, Parnak (2016) reported that the highest water content in the leaves was observed due to foliar application (500 ppm elicitor) which led to an agreeable amount of relative leaf water content, compared to the control.

Leaf catalase enzyme: The interaction effect of time showed that leaf catalase enzyme increased over time, up to the tenth day, compared to the third day, and that the most effective treatment was the 1000 ppm on the third day, which differed significantly from the negative control. Among the flowers sprayed with *B. cinerea* spores after elicitor treatment, on the tenth day, the highest elicitor concentration (1000 ppm) was the best concentration that could maintain leaf catalase, compared to the positive control. Measurement of catalase activity in the petals of several species such as chrysanthemums (Chakrabarty *et al.*, 2007) and cloves (Zhang *et al.*, 2007) showed that catalase activity in all these flowers increased during the development of buds into flowers. In many of these cases, there was a subsequent decrease in catalase activity through the aging phase. The reason for the increase in catalase activity is that the enzyme acts as one of the important components of the defense mechanism in plants.

Disease severity and leaf superoxide dismutase: The analysis of variance showed that the effect of treatments was significant ($P \leq 0.01$) on the occurrence of disease and its severity. The effect of treatments on the severity of leaf disease indicated that all three elicitor concentrations had a positive effect on plants, however, the best concentration was 1000 ppm, which was significantly different from the effect of other concentrations and the positive control (Fig. 1).

The interaction effects of elicitor and time significantly affected by the amount of leaf superoxide dismutase enzyme. It was observed that the amount of this enzyme increased over time through to the tenth day, compared to the third day, and that the most effective treatment was the elicitor at 1000 ppm. Among the flowers that were sprayed after elicitor treatment with *B. cinerea* spores, the 1000 ppm concentration was the best treatment that could better retain the content of leaf superoxide dismutase, compared to the positive control. The results showed that this enzyme's activity level in treated plants was much higher than in control conditions, which can indicate the effect of this enzyme in reducing oxidative stress damage. Saha and Pal (2021) revealed that superoxide dismutase was an important component of the defence mechanism in plants.

The treatment of flowers with fungal elicitors increased their vase life in the 'Samourai' cultivar by increasing the antioxidant capacity, reducing stress on flowers, controlling the negative effects of *B. cinerea* and maintaining the quality of flowers in general. Due to high importance and the extensive trade in roses, there is a need to increase the vase life of cut flowers and, thus, the current research served as an achievement in prolonging the vase life. According to the results of this study, the use of fungal elicitors at 1000 ppm can help maintain quality, reduce the negative effects of *B. cinerea* and increase the vase life of roses in the process of marketing.

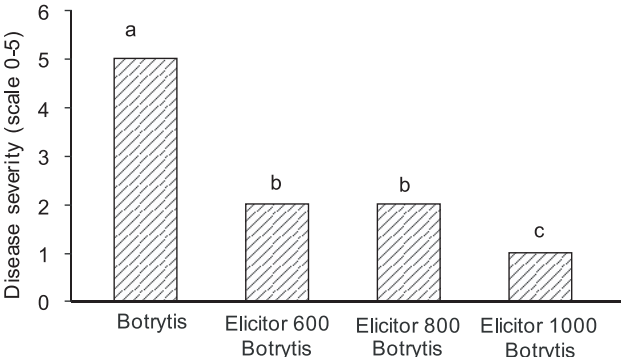

Fig. 1. Main effect of treatments on the disease severity index of rose cut flowers cv. 'Samourai'

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