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Evaluation of *Botrytis cinerea* isolates for virulence on rose cut flowers in greenhouse condition in Iran

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Gray mold of rose flowers (*Rosa hybrida*) is one of the well known and widespread diseases in the world. In 2006–2007, twelve infective isolates of *B. cinerea* on rose flowers from different provinces of Iran were tested on cut flowers *in vitro*. Fungal isolates cultured in Potato Dextrose Agar media and kept under 20±1°C as well as fluorescent light (1000 lux) conditions for 10 days. Then a suspension of 10^4 spores/ml from each fungal isolates was prepared as inoculum. Cut flowers of rose, Passion variety were inoculated in a way that each flower shared 1ml of spore suspension. Inoculated treatments were placed in bottles half filled with tap water and covered with plastic bags for 24 hours. Development of disease was studied from the third day after inoculation for each isolate by calculating disease index. Results showed significant differences among virulence of isolates from different regions of Iran. Among them Isolate-10 from Khuzestan and 2 from Gilan Provinces had the most and least amounts of virulence, respectively. Inoculated cut flowers with isolate 10 showed the disease symptoms 12 hours after inoculation while the disease progress was slower (at least 7 days) for the other tested isolates. Disease symptoms on cut flowers treated by isolate 2 only appeared as restricted tiny necrotic spots seven days after inoculation. In another experiment it was shown that the most appropriate inoculum concentration for *B. cinerea* is about 10^4 spores/ml.

Key words: *Botrytis cinerea*, gray mold, rose, virulence, Iran

Introduction

Since 4-5 thousand years ago roses are among the most important ornamental plants grown throughout the world (Kenneth, 1989). It is also the

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prevalent ornamental plant in Iran and more than 300 hectares of Khuzestan province lands especially in northern parts have been devoted for raising roses (Etaati, 2003). Gray mold of rose flowers with causal agent *Botrytis cinerea* Pers. Fr. is a destructive pathogen of roses grown as cut flowers in Iran (Ershad, 1995) as well as other countries (Hammer and Evensen, 1996; Tatagiba *et al.*, 1998). The pathogen colonizes petals causing lesions that reduce both yield and quality (Volpin and Elad, 1991). Infected petals show reduced growth and irregular lesions with red margin develop on them. The disease favours cold humid weather and under favourable condition a grayish brown mold layer is developed on infected flowers which include the asexual reproductive structures (conidiophores and conidia) of the Fungus (Etaati, 2003). Usually, intense economic loss occurs after harvesting, as undetected latent infections are established during the growing season. The latent infections may become severe in the wet, cool, and dark conditions commonly associated with storage and transport of cut roses (Hausbeck and Moorman, 1996). Such affected flowers then lose their market value (Elad, 1988; Hammer, 1988). Since different isolates of *B.cinerea* vary from each other in terms of producing cell wall degrading enzymes like pectinases, cutinases as well as cellulases thus they may show significantly different virulence towards their host plant (Kapat *et al.,* 1998).

Materials and methods

During 2006-2007, isolates of *B. cinerea* have been collected from different regions of Iran included two isolates from Gilan province, two isolates from Tehran province, three isolates from Markazi province and five isolates from Khuzestan Province. All the isolates have been cultured in Potato Dextrose Agar medium and incubated at 20ºC and fluorescent light (1000 lux) light to induce sporulation (Braun and Sutton, 1987). 7 to 9 day-old (Maximum 14 day-old) cultures were used for preparing spore suspensions. Each plate then rinsed three times with sterile distilled water and then suspensions with concentration of 10^4 spores/ml from each isolate were prepared. 500 cut rose flowers of Passion cultivar in the economically harvesting stage *i.e.* onset of bursting sepals were chosen from Safi Abad research center hydroponic green house which designated and built for this purpose. The cut flowers picked out in a way that they shared the same growth condition. In order to prevent microbial contaminations as well as clogging the vessels, basal part of rose stems up to 5 cm were placed for two hours in 8-hydroxyquinoline citrate (Teixeira daSilva, 2003). Then the cut flowers divided into groups of 12 cut flowers with 35cm. stem length to be inoculated by spore suspensions of the isolates. Inoculations were carried out by turn table method and the spore suspensions hand sprayed on the cut flowers in a way that each one shared 1 ml

of the suspensions. Some cut flowers also hand sprayed with distilled water as control treatment. The cut flowers then replaced one by one in bottles half full of tap water and covered by plastic bags immediately and then incubated at 20ºC. Twenty four hours later the plastics bags removed and the disease progress was examined for each isolate using Capdeville *et al.* method (Capdeville *et al.*, 2004). To calculate disease index, two different indicators *i.e.* Disease incidence and Disease severity have been studied simultaneously. For measuring disease severity in each treatment, cut flowers examined and scored according to following table (Capdeville *et al.,* 2004):

Disease severity (%)	0	0-2	2-5	5-10	10-15	15-25	25-50	50-75	75-100	100
Scores	1	2	3	4	5	6	7	8	9	10

Disease incidence was also determined separately for each treatment based on the number of flowers in 12 cut flowers that fall in to each disease severity categories. Disease Index was then calculated based on following formula where X and Y indicate the disease severity and disease incidence, respectively:

$$\text{Disease Index} = 100 \times \frac{(X1Y1)+(X2Y2)+(X3Y3)+\ldots\ldots(XnYn)}{Total}$$

Data were analyzed using Duncan multiple range test. Another experiment was also conducted to find out the most appropriate concentration of spore suspensions for pathogenicity tests. For this purpose, three different concentrations (10^2, 10^4 and 10^6 spores/ml) from fungal isolates showed intermediate disease severity. Selections were based on based on results of first experiment. These suspensions inoculated on cut flowers of Passion cultivars using the same method mentioned above.

Results

According to table 1 there are significant differences among the virulence of *B. cinerea* isolates. Isolates number 10 and 2, from Khuzestan as well as Gilan provinces had the most and the least virulence, respectively. Cut flowers inoculated by isolate number 10 showed the symptoms of the disease 10-12 hours after inoculation which first appeared as large chlorotic areas on the petals which immediately turned into brown necrotic blotches with gray centers. The rate of disease progress for the other isolates was slower. Completely developed blight (covering the infected tissues) appeared one week after inoculation. But those inoculated with isolate number 10, the necrotic spots frequently developed

and covered all the surfaces of the flowers in less than four days after inoculation. The affected flowers with isolate number 2, from Gilan province, showed least disease symptoms (appeared as single small necrotic spots on petals) after seven days of inoculation. Since two isolates from Gilan province were strictly of the same virulence, we omitted the isolate 1 from our analyzed data.

Intensity of the disease caused by highly virulent isolates was very higher than that of the less virulent ones, up to first three days after inoculation (Table 1). After that the rate of disease progress for the highly virulent isolates became lower in comparison to less virulent isolates. This was because of rapid colonization of all affected tissues with highly virulent isolates during first three days. So it seems that the first three days after inoculation could be a suitable criterion to compare and categorize the virulence of tested isolates. The disease progress curve as well as the rate of infection progress of isolates also showed the differences of the disease severity among isolates (Fig. 1).

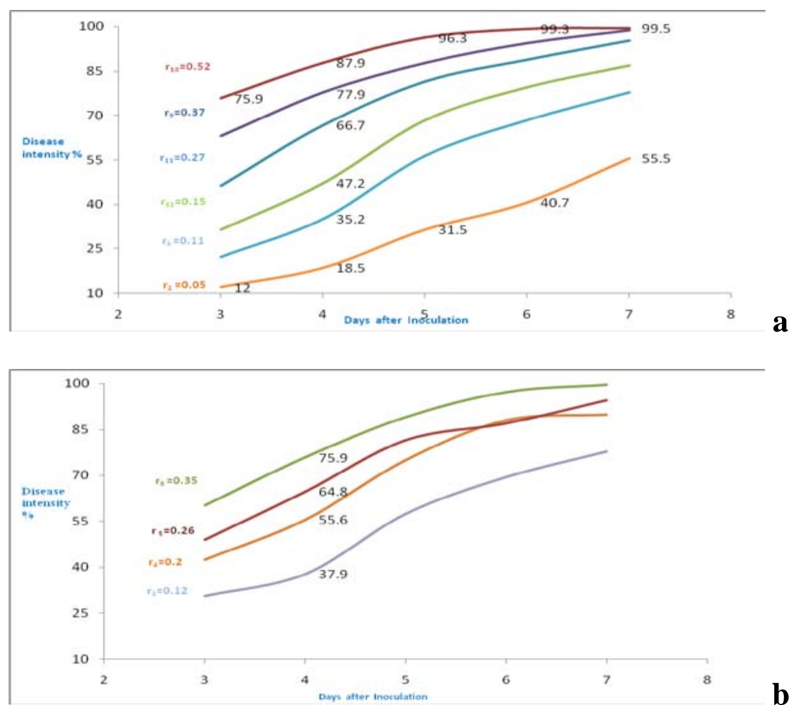

Fig.1. Disease progress curve of 6 from 11 tested isolates of *B. cinerea* on rose cut flowers. The most virulence isolate i.e. isolate 10 colonizes almost all the infected tissues after four days. To better understanding the differences among the intensity of infections occurred by these isolates the progress rates of each isolate (r) at fourth day after inoculation was studied. Note: since one cycle of the infection was studied the disease was treated as a monocyclic one (a), the rates of disease progress for other isolates at fourth day after inoculation (b).

In order to determine the appropriate concentration of inoculated spore, three different concentrations i.e. 10^2, 10^4 and 10^6spore/ml were tested. For this purpose, isolate number 8 from Khuzestan province was selected for preparing spore suspensions because of its mild severity compare to others. According to table 2, the best spore concentration for the inoculation of rose cut flowers was 10^4spore/ml. The disease had an extremely slow and high progress using suspension of 10^2 and 10^6 spore/ml., respectively. In the first case the infection process would take so long to be assessed correctly while in the latter case the rate of disease progress would be higher to let compare different isolates in terms of disease severity. Thus, separation of isolates based on their virulence would not be possible (Table 2).

Table 2. Effect of different spore concentrations of isolate number 8 on disease index. C1, C2 and C3 are representatives for 10^2,10^4 and 10^6spore/ml concentrations. ** indicates significant difference at 1% level.

Concentration Spore/ml	**Disease Index Per day**				
	Second	Third	Fourth	Fifth	Seventh
10^2	13.3 C	23.5 C	34.4 C	46.3 C	50.4C
10^4	26.8 B	40.0 B	51.1 B	63.0 B	65.5 C
10^6	54.6 A	63.2 A	71.6 A	77.6 A	79.8 A
F test	**	**	**	**	**

To study correlation of disease index with interactions between isolate and different spore concentration, another experiment was conducted where only four isolates *i.e.* isolates 3, 4, 7 and 8 with intermediate virulence were tested. The results showed that there is significant difference among different isolates when they inoculated with 10^4spore/ml on rose cut flowers (Table 3).

Discussion

The results of this research are approximately in accordance with Elad *et al.* (1988) where suspension of 10^4 spores/ml was the best one for causing the disease, examining different concentrations of spore on Rose flowers. On the other hand directing the same study Hammer and Morris (1989) concluded that suspension of 10^3 spores/ml could be the finest for inoculation of the flowers. The outcome of this experiment are also comparable with Kerssies *et al*., (1997) where five isolates of *B.cinerea* sampled inside and outside of a glasshouse for assessment of germination and pathogenicity. They showed differences of 66-99% and 14-166% among the examined isolates for the two

mentioned qualifications respectively. Milena and Silva (2005) isolated different strains of the fungus from tomato and grapevine plants and examined the pathogencity on tomatoes. Their results showed significant differences among the tested strains in terms of their virulence. They also showed their ability n producing variety of enzymes including polygalacturonases, methyl pectin esterases and so on.

Table 3. Interactions between isolates and spore concentrations vs. disease index per day after inoculation. C1, C2 and C3 are representatives for 10^2,10^4 and 10^6spore/ml concentrations.** indicates significant difference at 1% level. Means with the same letters had no significant differences at 1% level (LSD test).

Treatment	Disease Index per day				
	Second	Third	Fourth	Fifth	Seventh
Is. 8C3	70.1 B	81.5 A	95.1 A	96.3 A	100A
Is. 4C3	75.9 AB	86.4 A	94.5 A	98.1 A	100A
Is. 3C3	78.4 A	88.3 A	94.5 A	98.8 A	100A
Is. 7C3	77.8 AB	87.7 A	93.2 A	97.5 A	100A
Is. 8C2	46.3 C	60.5 B	74.1 B	88.3 AB	89.5 AB
Is. 4C2	34.6 D	65.4 B	75.3 B	82.7 B	83.5 B
Is. 7C2	45.1 E	58.6 B	69.1 B	82.7 B	84.0 B
Is. 8C1	19.1 EF	35.2 C	52.5 C	66.1 C	67.3 C
Is. 3C2	16.7EF	25.9 D	50.0 CD	59.3 CD	65.4 CD
Is. 4C1	16.7FG	25.9 D	40.1 DE	52.5 DE	58.0 CDE
Is. 3C1	13.O FG	23.6 DE	37.O E	53.1 DE	54.3 DE
Is. 7C1	12.3 FG	26.5 D	37.7 E	46.7 EF	56.2 CDE
Is. 6C3	16.1 EF	22.8 DE	35.2 E	49.4 DE	52.5 EF
Is. 6C2	11.1 FGH	17.3 EF	23.5 F	35.8 FG	41.4 FG
Is. 6C1	8.6 GH	16.7 EF	24.1 F	35.8 FG	40.1 GH
control	9.9 GH	13.0 F	14.8 F	23.5 H	26.5 I
control	6.8 H	12.3 F	14.8 F	29.0 GH	29.0 HI
control	8.6 GH	12.3 F	17.3 F	25.3 GH	26.51
F test	**	**	**	**	**

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Table 1. Individual effect of tested *B.cinerea* isolates on disease index per day after inoculation. Note that there are no significant differences at 5% level among the means of the data with the same letters in each column (Duncan Multiple range test).

Isolates	Disease Index per day									
	Third		Fourth		Fifth		Sixth		Seventh	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
Is. 10	3.7	75.9 A	4.6	87.9 A	4.6	96.3 A	5.6	99.3 A	5.6	99.5 A
Is. 8	0.9	60.2 B	1.9	75.9 B	1.9	88.9 B	3.7	97.2 AB	4.6	99.5 A
Is. 9	1.9	63.0 B	3.7	77.9 B	3.7	87.9 B	4.6	94.4 BC	4.6	98.7 A
Is. 11	2.8	46.3 C	2.8	66.7 C	2.8	81.5 BC	4.6	88.9 CD	4.6	95.4 A
Is. 4	3.7	42.6 C	3.7	55.6 D	3.7	75.0 CD	3.7	87.9 CDE	3.7	89.8 BC
Is. 5	2.8	49.1 C	3.7	64.8 C	3.7	81.5 BC	3.7	87.0 CDE	3.7	94.4 AB
Is. 7	0.9	44.4 C	2.8	63.0 CD	2.8	81.5 BC	3.7	86.1 DE	3.7	89.8 BC
Is. 12	3.7	31.5 D	3.7	47.2 E	3.7	68.5 D	3.7	79.6 EF	3.7	87.0 BC
Is. 3	0.9	30.6 D	4.6	37.9 F	4.6	57.4 E	4.6	69.4 F	5.6	77.8 C
Is. 6	0.9	22.2 E	1.9	35.2 F	1.9	56.5 E	2.7	68.5 F	3.7	77.8 C
Is. 2	1.9	12.0 F	2.8	18.5 G	2.8	31.5 F	3.7	40.7 G	3.7	55.5 D