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Inducing Resistance to Thiophanate Methyl in *Penicillium expansum* through Mutagenesis

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Abstract

Isolation of *Penicillium expansum* from diseased pear fruits and their sensitivity were evaluated and identified as resistant and sensitive. Further research involved the utilization of susceptible *Penicillium expansum* isolates. Sensitive isolate (Pe-11) conidia of *Penicillium expansum* were subjected to thiophanate methyl and application of various physical mutagenic treatments, including spontaneous mutation (Sp), UV lamp, and chemical mutagens such as Bromyl Uracil (BU), Ethyl Methane Sulphonate (EMS), and N-Nitro-N-Methyl Urea (NMU), to the sensitive isolate of *Penicillium expansum* (Pe-11) revealed the emergence of mutants with varying resistance levels. Among them, highly resistant mutants demonstrated exceptional virulence, while moderately resistant and less resistant mutants also exhibited degrees of virulence. It was observed that UV ray treatment yielded a fruitful percentage of mutants, followed by SP, NMU, and EMS. The study concentrated on formulating fungicide resistance in resistant and sensitive isolates, emphasizing spontaneous mutation, UV, BU, EMS, and NMU. This investigation indicated a notable rise in the frequency percentage and a greater number of mutants compared to alternative treatments. Furthermore, the highest pathogenicity was attributed to mutants obtained via spontaneous, UV, and EMS treatment.

***Key words*:** Thiophanate methyl, *Penicillium expansum*, Mutagens, Resistant, Pear

Penicillium expansum Link. Thom.ex., the causative agent of blue mold in pears, stands out as one of the most severe post-harvest diseases globally, including in India. As a result, a study of post-harvest maladies affecting pear fruits under market storage conditions in Maharashtra was undertaken in order to develop effective management strategies. *Penicillium expansum* was identified as the prevailing post-harvest pathogen in storage areas of central and local fruit markets across various locations in Maharashtra, with a particular emphasis on the APMC fruit market in Vashi, Navi Mumbai where various damages were observed in pear packaging cases. Preharvest and post-harvest maladies of various fruits may reportedly be controlled with conventional and systemic fungicides, such as thiophanate methyl.

Twenty-three *Penicillium expansum* isolates were obtained from pear specimens infected with the fungus to assess their sensitivity to thiophanate methyl on the Potato Dextrose Agar (PDA) medium. The food poisoning technique was employed to ascertain the susceptibility of these isolates to thiophanate methyl [1]. Induction of thiophanate methyl resistance in *Penicillium expansum* through certain physical and chemical mutagens viz. spontaneous mutation (SP), Ultraviolet (UV) lamp, Bromyl Uracil (BU), Ethyl Methyl Sulphonate (EMS), and N-Nitro-N- Methyl Urea (NMU). Classification of mutants was settled based on the severity of thiophanate methyl resistances, i.e., highly resistant (HR), moderately resistant (MR), and sensitive (S). The highly resistant factor went up to 5 (*Pe*-EMS-11). The development of resistance may also affect the virulence of the pathogen. Hence, a comparison between resistant and sensitive isolates of blue mold of pear was also made. The resistant strain appeared to be more virulent.

On the other hand, genetic changes caused by stable fungicides in the pathogen are more important. This development of resistance depends upon the number of mutations required for resistance and mutability of the gene at the loci concerned. It may be influenced by the type of fungicide, type of pathogen, and environmental conditions, e.g., UV or other radiation [2]. Genetic resistance may be due to the inhibitory activities of biosynthesis [3]. Fungicide resistance is attributed to a modification at the site of action, which decreases the affinity between the chemical and its site [4]. The protectant fungicides act as multisided inhibitors, so if mutational resistance occurs, it is unlikely at all sites. Systemic fungicides are characterized by their ability to penetrate plant tissues and exert their effects at specific sites within the fungal cell metabolism, offering targeted and efficient control against a limited number of fungal processes.

MATERIALS AND METHODS

A culture of *Penicillium expansum* was isolated from the blue mold of the pear and maintained on PDA slants. Spores were resuspended in sterile distilled water to treat UV-

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mutagenesis after being cultured for 5 days. They were then exposed to a UV-light (254 nm) source at a distance of 25 cm for the following durations: 10, 15, 20, 25, and 30 minutes, before being moved to dishes containing Potato Dextrose Agar medium, the treated conidia were left in darkness for an hour. Alternatively, chemical mutagens such as EMS (Ethyl Methane Sulfonate), NMU (N-Nitro-N-Methyl Urea), and BU (Bromyl Uracil) mutagenesis were applied to the conidia of 5-days-old cultures. A solution containing 100-400µg/ml of EMS, NMU, and BU was used to incubate the collected spores for 30 minutes in a phosphate buffer with a pH of 7.0. After rinsing the spores with distilled water to remove any contaminants, they were diluted in a series of steps to inoculate the Potato Dextrose Agar medium.

Mutants and the sensitive strain were cultured in a PDA medium. Spore suspensions at a concentration of 10% were introduced into the media. One hundred milliliters of liquid were used to cultivate the spores of *Penicillium expansum*. Spores were propagated using a method outlined by Horsten [5]. After sterilization, the plates were filled with the PDA medium that included (3X-MIC) of thiophanate methyl. The mixture was then let to harden. Using an L-shaped sterile glass rod, one of the spore suspensions was evenly spread over the surface of the PDA media. The resulting Petri dishes were then incubated at 27±2ºC for a duration of 10 days. After counting the visible colonies, we determined the percentage frequencies of resistant colonies by comparing them to the control plates. Colonies exhibiting different morphological characteristics were then segregated and kept on a PDA medium without fungicide for future research. It was determined that the colonies exhibiting distinct morphological traits were UV mutants after they were moved to a PDA medium devoid of fungicide. Various morphologically diverse colonies were isolated on fungicide-free PDA media. Members of the mutant population were labeled as BU-mutants, EMS-mutants, and NMU-mutants. Post-harvest fruits of pear were surface sterilized with 10% sodium hypochlorite solution and washed 5-10 times with sterile distilled water. The organisms were exposed to a spore slurry containing *Penicillium expansum* isolates or mutants that were either sensitive or resistant to thiophanate methyl. A Percentage Control Efficacy (PCE) estimate was performed [6]. Research on the efficacy of thiophanate methyl and other fungicides was conducted by employing a tissue paper method of fruit wrapping. The following grades of infection on the fruits' skin were determined after 15 days of attentive handling.

Numerical rotting	Fruits area showing an infection
0	Fruits healthy
1	1 to 25% area infected
2	26 to 50% area infected
3	51 to 75% area infected
4	76 to 100% area infected

RESULTS AND DISCUSSION

Results revealed (Table 1) that UV gave a higher percentage frequency of mutation and a higher number of mutants. This was followed by EMS, spontaneous, and Bromyl Uracil. Bromyl Uracil mutagen was observed, and 62 mutants were obtained. For stability testing, the mutants were reisolated on potato dextrose agar slants. This medium included 3X MIC of thiophanate methyl, and they were inoculated on it again after being transferred 10 times on standard PDA. Those mutants served, and growths were considered stable mutants (Table 2). It was confirmed once more that the mutants obtained through UV and EMS treatments exhibited significantly higher sensitivity to thiophanate methyl resistance compared to the fewer mutants obtained from other treatments like spontaneous, NMU, and BU. The higher resistant factor of pathogenicity of thiophanate methyl resistant mutant of *Penicillium expansum* obtained through EMS treatment and resistant factor went up to 5 (i.e., Highly resistant) and percentage disease index, i.e., 42.75 (Table 3).

Table 1 Induction of resistance to thiophanate methyl in *Penicillium expansum* through various mutagenic treatments

S. No.	Treatment time (min)	No. of mutants	Percentage of frequency
1.	Spontaneous	28	46.66
2.	UV rays (30 min)		
	10 min	37	61.66
	15 min	35	58.33
	20 min	33	55.00
	25 min	30	50.00
	30 min	28	46.66
3.	Bromyl Uracil (30 min) (BU)		
	1) 100 µg/ml	19	31.66
	2) 200 µg/ml	17	28.33
	3) 300 µg/ml	15	25.00
	4) 400 µg/ml	11	18.33
4.	Ethyl Methane Sulphonate (30 min) (EMS)		
	1)100 µg/ml	20	33.33
	2) 200 µg/ml	22	36.66
	3) 300 µg/ml	25	41.66
	4) 400 µg/ml	24	40.00
5.	N-Nitro-N- Methyl Urea (30 min) (NMU)		
	1) 100 µg/ml	22	36.66
	2) 200 µg/ml	20	33.33
	3) 300 µg/ml	22	36.66
	4) 400 µg/ml	20	33.33

Table 2 Stability of *Penicillium expansum* mutants resistant to thiophanate methyl

S. No.	Nature of mutant	Total mutants tested	Stable mutants	Stability percentage
1.	Spontaneous	28	09	33.14
2.	UV rays (30 min)			
	10 min	37	10	27.02
	15 min	35	09	25.71
	20 min	33	10	30.30
	25 min	30	08	26.66
	30 min	28	07	25.00
3.	BU (30 min)			
	1). 100 µg/ml	19	05	26.31
	2). 200 µg/ml	17	04	23.52
	3). 300 µg/ml	15	05	33.33
	4). 400 µg/ml	11	04	36.36
4.	EMS (30 min)			
	1). 100 µg/ml	20	08	40.02
	2). 200 µg/ml	22	06	27.28
	3). 300 µg/ml	25	07	28.01
	4). 400 µg/ml	24	07	29.16
5.	NMU (30 min)			
	1). 100 µg/ml	29	08	27.58
	2). 200 µg/ml	25	06	24.07
	3). 300 µg/ml	22	06	27.27
	4). 400 µg/ml	20	05	25.00

The mutant was subcultured 10 times on plain potato dextrose agar and again cultured on PDA containing 3X MIC of thiophanate methyl

Table 3 Resistance factor of pathogenicity of thiophanate methyl resistant mutant of *Penicillium expansum* obtained through induced mutations

S. No.	Mutants	Resistant factor	Resistance category	PDI %
1.	Pe-Sp-1	2	R	18.50
2.	Pe-Sp-3	2	R	20.00
3.	Pe-Sp-5	2	R	18.5
4.	Pe-UV-1	4	MR	23.75
5.	Pe-UV-4	3	R	18.50
6.	Pe-UV-5	3	R	20.00
7.	Pe-BU-1	2	R	20.00
8.	Pe-BU-2	3	R	18.50
9.	Pe-BU-4	2	R	20.00
10.	Pe-BU-6	3	R	18.50
11.	Pe-EMS-3	3	R	20.00
12.	Pe-EMS-4	2	R	18.50
13.	Pe-EMS-6	3	R	18.50
14.	Pe-EMS-7	3	R	18.50
15.	Pe-EMS-10	4	MR	23.75
16.	Pe-EMS-11	5	HR	42.75
17.	Pe-EMS-12	4	MR	23.75
18.	Pe-NMU-3	3	R	18.50
19.	Pe-NMU-6	3	R	20.20
20.	Pe-NMU-7	4	MR	23.75
21.	Pe-Wild sensitive	S/300	S	12.5

A: MIC of thiophanate methyl against resistance mutant divided MIC of wild Sensitive isolate. Resistant factor 2-3, Moderately Resistance – 4, Highly Resistant -5

B: HR- Highly Resistance, MR- Moderately Resistance, R- Resistant, and S- Sensitive to thiophanate methyl

Table 4 Sensitivity of *Penicillium expansum* isolates from saltation plate against thiophanate methyl

S. No.	Treatments	ED_{50} (µg/ml)	MIC (µg/ml)	RF
1.	Pe-sp-1	0982	1966.9	3.022
2.	Pe-sp-3	0970	1941.8	2.983
3.	Pe-sp-5	0986	1971.6	3.030
4.	Pe-UV-1	1941	3884.5	5.971
5.	Pe-UV-4	1457	2915.6	4.482
6.	Pe-UV-5	1459	2910.8	4.474
7.	Pe-BU-1	0974	1946.4	2.992
8.	Pe-BU-2	1455	2911.5	4.475
9.	Pe-BU-4	0978	1943.5	2.987
10.	Pe-BU-6	1459	2919.4	4.487
11.	Pe-EMS-3	1460	2910.2	4.473
12.	Pe-EMS-4	9730	1964.1	3.019
13.	Pe-EMS-6	1452	2913.2	4.478
14.	Pe-EMS-7	1455	2919.4	4.487
15.	Pe-EMS-10	1940	3880.2	5.964
16.	Pe-EMS-11	2426	4921.5	7.466
17.	Pe-EMS-12	1942	3886.4	5.974
18.	Pe-NMU-3	1459	2919.2	4.487
19.	Pe-NMU-6	1457	2921.5	4.491
20.	Pe-NMU-7	1944	3890.2	5.978
21.	Pe-11	290.6	0650.5	1.000

ED_{50}: Fungicide concentration causing a 50% reduction in radial growth

MIC: Minimal inhibitory concentration

RF: Resistance factor

While observing the plates, the saltation (sectors in the colony) was noted. Twenty isolates were obtained from each sector and were tested for their sensitivity against thiophanate methyl as usual. There was bound to be a notable variation in ED_{50} and MIC of thiophanate methyl against *Penicillium expansum* isolates. The MIC ranged from 1941.8-4921.5µg/ml. Pe-sp-3 was the most sensitive isolate, while Pe-EMS-11 was a resistant mutant. Other isolates showed their sensitivity between 1966.9-3890.2µg/ml. The saltation phenomenon may help in the development of fungicide resistance in *Penicillium expansum* (Table 4). The present study examined the induction of thiophanate methyl resistance in the sensitive *Penicillium expansum* isolate using Spontaneous (Sp), Ultraviolet (UV), Bromyl Uracil (BU), Ethyl Methane Sulphonate (EMS), and N-Nitro-N-Methyl Urea (NMU). EMS produced a higher percentage of frequency, quantity of mutations, stability, and pathogenicity than previous treatments. Among these treatments, EMS was found to be particularly effective.

Sable and Gangawane [7] reported that the induction of carbendazim resistance in *Aspergillus niger* was most pronounced with EMS treatment, yielding the highest number of mutants, followed by UV, BU, and Sp treatments. Van Tuyl [8] induced the benomyl and thiobendazole resistance by UV treatment in *Penicillium expansum*, *Aspergillus nidulance*, and *Cladosporium caumerinum*. Ushiyama and Davidse [9-10] observed that *Aspergillus flavus* treated with EMS and UV developed carbendazim and thiophanate methyl mutants, which led to thiophanate methyl resistance in *Penicillium digitatum* and *Penicillium italicum*. Because of this, the EMS was stronger. However, it should be emphasized that the emergence of resistance mutants in the laboratory due to these compounds does not imply in the field because it leads to control failure. This determination only happens after a considerable proportion of the pathogen population has become resistant. Moreover, the present study may be of more help in forecasting the resistance development in the *Penicillium expansum* isolate against thiophanate methyl. Similar results were also reported in agreement with Dekker, Gangawane, Reddy, Reddy, Khilare, and Gangawane, Suryawanshi [11-15].

CONCLUSION

In laboratory settings, the degree of fungicide resistance in a pathogen can be manipulated through the induction of resistance using diverse physical and chemical mutagenic treatments. This approach could aid in effectively managing fungicide resistance in a given pathogen. In the present investigation, induction of thiophanate methyl resistance in the sensitive *Penicillium expansum* isolates through the treatment of Spontaneous (Sp), Ultraviolet (UV), Bromyl Uracil (BU), Ethyl Methyl sulphonate (EMS) and N-Nitro-N- Methyl Urea (NMU). Comparatively, EMS produced greater frequency, number of mutations, stability, and pathogenicity results. An investigation into the emergence of fungicide resistance in sensitive and resistant isolates revealed that the interventions comprising spontaneous mutation, ultraviolet light, BU, EMS, and NMU generated the greatest number of mutants and had the highest percentage frequencies. The study's results demonstrate a significant correlation between mutations obtained through UV, EMS, and spontaneous means and an increased propensity for disease.

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