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In vitro screening method: An efficient tool for screening *Alternaria* blight resistance/tolerance during early generations in Ethiopian mustard (*Brassica carinata* A. Braun)

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Rapeseed-mustard crops in general, show low average productivity due to the prevalence of various biotic and abiotic stresses. Among biotic stresses, diseases such as white rust and *Alternaria* blight are the major contributing factors. *Alternaria* blight caused by *Alternaria brassicae* has been reported to cause variable losses in yield. The present study aimed to induce mutations for *Alternaria* blight resistance/tolerance in Ethiopian mustard and screen the induced mutants through *in vivo* (detached leaf method) as well as *in vitro* (cultural filtrate) methods for disease resistance/tolerance in different generations. About 46 mutants in M_2 generation were isolated which showed segregation for *A. brassicae* tolerance. Only 10 mutants showed very less sporulation intensity along with less halo and concentric ring diameter. These mutants were further evaluated under natural field conditions at Kangra to confirm their reaction. Out of these, only two mutants viz., P $(_4)_2$ in 80 kR and P_{13} in 100 kR doses were observed to be moderately resistant/tolerant against *A. brassicae* (PDI < 25.0%, scale 2). The behaviour of theses mutants was further confirmed by *in vitro* studies. Both mutants showed pale yellow to light brown and fragile callus in all three concentrations of fungal filtrate. Both fresh and dry weights of calli were maximum in 80 and 100 kR dose-explants in M_0 and M_4 generations as compared to 50, 60, 70, 90, and 110 kR dose-explants in M_0 generation. The *in vitro* selection could effectively be used to screen genotypes right in M_0 generation itself as 80 and 100 kR doses exhibited moderate resistance/tolerance against *Alternaria* blight both in M_0 and M_4 generations. Hence, *in vitro* selection method can be used as an efficient tool for screening mutants dose-wise in initial generations, saving time and laborious field work required to screen mutants *in vivo* for a minimum of 3 to 4 generations.

Key words: *Alternaria blight, brassica,* callus, cultural filtrate, *in vivo, in vitro,* resistance.

INTRODUCTION

The accessibility of sufficient genetic variability and efficient selection procedures are two essential fundamentals in plant breeding. Genetic variability can be broadened by utilizing wild and related species in

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can be obtained through mutation induction techniques or somaclonal variation. The latter two methods are particularly useful if the desirable trait is not present in wild or related species and/or the same cannot be easily introgressed through conventional breeding. Mutants and somaclonal variants can be selected, for instance, for disease resistance by using an appropriate selection agent (potentially derived from a pathogen). DNA alterations viz., mutations that affect the gametes, can be transmitted to the progeny thus; the selected plant material is a new valuable source of genetic variability for the improvement of resistance to pathogens. A basic knowledge about the biology of the causal agent and its relationship with the host plant is necessary for the development of suitable methods of screening and selection for resistance (Russell, 1978). There is a broad range of different approaches available to detect resistant genotypes and select plants with improved resistance (Singh and Singh, 2005). Among these, *in vitro* screening is one of the most efficient methods (Švábová and Lebeda, 2005). Tissue culture or *in vitro* methods are being used effectively in many basic and applied areas of research in plant biology. The possibility of selecting plants for resistance in cultures *in vitro* was probably demonstrated for the first time by Carlson (1973). Since then, substantial progress has been made in this field yielding a large number of *in vitro* selected germplasm with the potential for developing disease resistant plants (Daub, 1986) whereas traditional methods of screening for disease resistance are quite cumbersome, time consuming and success remains at the mercy of natural conditions.

Rapeseed-mustard crops are the most important *rabi* oilseed crops grown in India. In general, the crops showed low average productivity due to the prevalence of various biotic and abiotic stresses. Among biotic stresses, diseases are the major contributing factors. *Alternaria* blight caused by *Alternaria brassicae* (Berk.) Sacc. is an important and a widespread destructive disease of rapeseed-mustard (Weiss, 1983). The disease has been reported to cause variable losses in yield. Depending upon its severity in India, the yield losses to the extent of 70% have been reported (Saharan, 1991). To curtail the huge losses in yield, development of resistance to pathogen has become an important factor in rapeseed-mustard breeding programmes. Therefore, keeping in view the above, the present study aims to induce mutations for *Alternaria* blight resistance/tolerance in Ethiopian mustard and screen the induced mutants through *in vivo* as well as *in vitro* methods for disease resistance/tolerance in different generations.

MATERIALS AND METHODS

For the induction of mutations, the dry and uniform sized seeds of variety 'Jayanti' (a mutant of HC-1 induced through irradiation) were exposed to 50, 60, 70, 80, 90, 100 and 110 kR of gamma radiations to induce resistance/tolerance to *Alternaria* blight, at Bhabha Atomic Research Centre, Trombay, Mumbai. M_2 to M_4 generations following physical mutagens were evaluated under natural field as well as artificial epiphytotic conditions at CSKHPKV, Shivalik Agricultural Research and Extension Centre, Kangra (hot spot for the disease-brief description of location is given below) in order to select desired disease resistant/tolerant mutants (*in vivo selection)*. On the other hand, *in vitro* selection of mutants in M_0, M_2, and M_4 generations was also carried out under laboratory conditions. Based upon the mean observations, a comparison for the similarities between the resistant/tolerant mutants *in vivo* (whole plant-pathogen interactions) and *in vitro* screening methods was also made.

Kangra

The experimental farm of CSKHPKV, Shivalik Agricultural Research and Extension Centre, Kangra is situated at 32°8'N latitude and 76°3'E longitude at an elevation of 700 m above sea level. Agroclimatically, the location represents the mid-hill zone of Himachal Pradesh (Zone II) and is characterized by subtemperate climate with 250 to 500 mm rainfall. The soil is clay-loam with pH 6.4.

In vivo studies on reaction to *Alternaria* blight

Single plant selections were made in M_2 generation of physically irradiated populations of parent 'Jayanti' under field conditions at Kangra. The field plots of CSKHPKV, SAREC, Kangra were already sick plots but, in order to avoid any escape, plots were inoculated with *in vitro* multiplied inoculum of virulent isolates of *A. brassicae* (during both vegetative and reproductive stages. Severity of *Alternaria* blight was recorded on five randomly selected leaves of each plant using 0 to 6 scale. Disease severity on each plant was converted into Percent Disease Index (PDI) (McKinney 1923) which facilitated the easy selection of resistant/tolerant plants. For generation advancement, the M_3 generation was raised in off-season nursery located at CSKHPKV, HAREC, Kukumseri (Lahaul and Spiti) during May, 2010. M_4 mutants were again evaluated under natural field conditions at Kangra during *rabi*, 2010 to 2011 to confirm their reaction against *A. brassicae*. As field plots were already sick plots but, in order to avoid any escape, the plots were inoculated with *in vitro* multiplied inoculums of virulent isolates of *A. brassicae* during both vegetative and reproductive stages. Severity of *Alternaria* blight was recorded on leaves of each plant as done earlier using 0 to 5 scale, by Detached Leaf technique (Bansal et al*.,* 1990) and by measuring size of naturally occurring concentric rings which facilitated the easy selection of resistant/tolerant mutants.

In vitro studies on reaction to *Alternaria* blight

This technique can be used efficiently to select disease resistant/tolerant genotypes by *in vitro* selections. Such an evaluation of disease resistance/tolerance is dependent upon the positive correlation between *in vitro* resistance to Cultural Filtrate (CF) and whole plant disease resistance (Willmot et al., 1989). The most virulent *A. brassicae* isolate $A.B_n$ was screened for its virulence and sub cultured on Potato Dextrose Agar (PDA). Small bits (5 mm diameter) of *A. brassicae* culture were transferred to the conical flasks (250 ml) containing 30 ml of sterilized Potato Dextrose Broth (PDB) and incubated at 22±1°C for 15 days in a dark incubator. Fungal mycelium was separated by passing fungal culture through sterilized Whatmann No.1 filter paper. The cultural

Table 1. Frequency of *Alternaria* blight resistant/tolerant mutants with desirable agronomically characters in the M_2 generation.

Treatment	Total M_2 progenies	Progenies egregating for *Alternaria* blight resistance/tolerance	%	Resistant/tolerant segregating progenies with other desirable characters	%
50 kR	1921	10	0.5	6	60.0
60 kR	2127	6	0.3	3	50.0
70 kR	1611	6	0.4	3	50.0
80 kR	1190	1	0.1	1	100.0
90 kR	1129	4	0.4	3	75.0
100 kR	867	5	0.6	4	80.0
110 kR	287	1	0.3	0	0.0
0.3 % EMS (PS)	2472	11	0.4	7	63.6
0.4 % EMS (PS)	1947	1	0.1	0	0.0
0.5 % EMS (PS)	1831	1	0.1	1	100.0

filtrate was further sterilized by passing through Millipore filter of 0.22 µm size and the pH was adjusted to 5.8 before filter sterilization. The crude concentrated fungal liquid cultural filtrate was mixed to the autoclaved MS medium along with hormonal combination viz; BAP-2.0 mg/l + NAA-0.1 mg/l during cooling under aseptic conditions at 20, 60, and 100% concentration of fungal filtrate and dispensed in sterilized petriplates. The medium was stored at 25 ± 1°C and used for inoculation. The hypocotyl segments of 5 to 6 mm length were excised from 8 to 10 days old seedlings of different doses viz., M_0 generation (50, 60, 70, 80, 90, 100, and 110 kR) and M_4 generation mutants that showed moderate resistance through *in vivo* screening along with the control Jayanti. These hypocotyl segments were implanted on MS medium (BAP-2.0 mg/l + NAA-0.1 mg/l) with or without cultural filtrate. The petri plates were sealed with Parafilm and incubated at a temperature of 25 ± 1°C for one month under 16 h light (1500 lux) and 8 h dark cycles. In each petri plate, 10 explants were cultured and whole experiment was replicated twice. After one month, the observations were recorded on percent callus induction, colour of callus, fresh and dry weights of individual calli and shooting response to screen for their resistance/tolerance.

RESULTS AND DISCUSSION

In vivo studies

Various M_2 progenies were screened under natural field conditions at Kangra for their reaction to *A. brassicae*. About 46 progenies were isolated which showed segregation for *A. brassicae* tolerance. Out of these, 28 progenies also exhibited other desirable characters coupled with *A. brassicae* tolerance (Table 1). Out of these, 45 mutants in M_4 generation were again raised at Kangra, for screening against *Alternaria* blight resistance/tolerance under both natural and artificial epiphytotic conditions under field as well as laboratory conditions. Under laboratory conditions, Detached Leaf technique was followed using the most virulent isolate from *B. napus* ($A.B_n$) (Kumari et al., 2013). The maximum incubation period (4.0 to 4.5 days) was recorded by 17 mutants but, only 10 mutants showed very less sporulation intensity along with less halo and concentric ring diameter. These mutants viz; P_7 (50 kR), $P(_{10})_2$ (50 kR), P_{11} (50 kR), P_{74} (50 kR), P_2 (70 kR), $P(_4)_2$ (70 kR), $P(_5)_2$ (70 kR), $P(_4)_2$ (80 kR), P_{22} (90 kR) and P_{13} (100 kR) were found to exhibit moderate resistance. These were further evaluated under field conditions at Kangra to confirm their reaction. Out of these, only two mutants such as P $(_4)_2$ in 80 kR and P_{13} in 100 kR doses were observed to be moderately resistant/ tolerant against *A. brassicae* (PDI < 25.0%, scale 2) (Table 2).

In vitro studies

Callus initiation

Callus initiation was observed within one week from cultured hypocotyl explants in almost all media (media without cultural filtrate and with 20 and 60% cultural filtrate), except in medium with 100% cultural filtrate. Explants cultured in this medium showed expansion after one week. Different dose-explants (both in M_0 and M_4 generations) had different response in all four media. Results revealed that, the early callus initiation and better callus growth which was indicated through percent callus induction, reduced drastically from lower to higher concentrations of cultural filtrate that is, 20 to 100% CF. This is because, the higher concentration of cultural filtrate inhibited cell division and caused death to cultured cells. However, 80 and 100 kR dose-explants (both in M_0 and M_4 generations) showed greater extent of survival in all concentrations of fungal toxin viz, 20, 60, and 100% cultural filtrate as compared to control Jayanti (parental explants). In addition to callus induction, color of callus also supported the above results. The selected mutants viz., P $_{(4)2}$ (80 kR) in M_4 generation showed pale yellow color and fragile callus in all three concentrations of fungal filtrate. Although, P_{13} (100 kR) showed light brown callus in 60 and 100% cultural filtrate, but, calli

Table 2. Response of 45 putative mutants in M4 of Brassica carinata to the A. brassicae through Detached Leaf technique.

B. carinata mutants	Dose	Mean incubation period (day)	Mean halo diameter (mm)	Mean concentric ring size (mm)	Sporulation intensity	Category
P_7	50 kR	4.5	40	38	Very less	MR
$(P_{10})_2$	50 kR	4.0	37	37	Very less	MR
P_{10}	50 kR	3.0	68	60	Da$rk	HS
P_{46}	50 kR	3.9	64	56	Dark	S
$(P_8)_2$	50 kR	4.2	42	41	Medium	MS
P_{11}	50 kR	3.7	40	40	Very less	MR
P_{74}	50 kR	4.0	45	42	Very less	MR
$(P_{11})_2$	50 kR	4.4	48	40	Medium	MS
P_{56}	50 kR	3.8	56	49	Dark	S
$(P_3)_2$	50 kR	3.6	73	61	Dark	S
P_{47}	50 kR	4.2	57	50	Dark	S
P_{26}	60 kR	3.8	65	55	Medium	MS
P_{39}	60 kR	3.5	56	51	Medium	MS
P_{38}	60 kR	3.0	75	68	Very dark	HS
$(P_1)_2$	60 kR	3.8	63	58	Sparsely	MS
$(P_9)_2$	60 kR	3.9	65	55	Dark	S
$(P_4)_2$	70 kR	4.4	45	36	Very less	MR
$(P_5)_2$	70 kR	4.5	48	45	Very less	MR
P_6	70 kR	3.7	78	70	Dark	S
P_{22}	70 kR	4.0	58	50	Medium	MS
$(P_2)_2$	70 kR	4.2	50	47	Medium	MS
P_2	70 kR	4.0	47	43	Very less	MR
$(P_4)_2$	80 kR	4.4	45	36	Very less	MR
P_{15}	90 kR	3.0	72	70	Dark	HS
P_2	90 kR	4.0	66	50	Medium	MS
P_{22}	90 kR	4.5	35	35	Very less	MR
$(P_2)_2$	90 kR	4.0	50	38	Medium	MS
P_{22}	100 kR	3.9	58	50	Medium	MS
P_4	100 kR	3.7	56	49	Medium	MS
$P_1)_2$	100 kR	3.8	60	55	Medium	MS
$(P_{13}$	100 kR	4.3	42	40	Sparsely	MR
$(P_2)_2$	110 kR	3.5	68	54	Medium	S
$(P_2)_2$	0.3% EMS-PS	3.0	75	70	Very dark	HS
P_{34}	0.3% EMS-PS	3.0	72	61	Very dark	HS
P_{31}	0.3% EMS-PS	3.0	74	60	Medium	S
P_{13}	0.3% EMS-PS	3.7	60	52	Medium	S
$(P_9)_2$	0.3% EMS-PS	3.1	68	59	Dark	S
$(P_{17})_2$	0.3% EMS-PS	4.0	50	45	Medium	MS
P_{27}	0.3% EMS-PS	3.0	56	50	Medium	S
P_{14}	0.3% EMS-PS	3.5	54	50	Very less	MS
P_{18}	0.3% EMS-PS	3.2	58	52	Sparsely	MS
P_5	0.3% EMS-PS	3.0	69	65	Very dark	S
$(P_{23})_2$	0.3% EMS-PS	3.0	74	70	Very dark	HS
$(P_1)_2$	0.4% EMS-PS	3.1	71	67	Dark	HS
P_8	0.5% EMS- PS	3.5	62	55	Medium	S

MR-Moderately resistant, MS-moderately susceptible, S-susceptible, HS-highly susceptible, EMS-PS-ethyl methane sulphonate with pre-soaking.

tenderness and other parameters indicated moderate resistance/tolerance against *Alternaria* blight (Figure 1). However, earlier workers (Kiran et al., 2002) observed the compactness of callus coupled with dark color on medium supplemented with higher concentrations of CF > 15%. They indicated that the ability of callus to grow in the presence of cultural filtrate of *A. brassicae* was related to whole plant response to the pathogen which could be exploited to know resistance/tolerance in *brassica* genotypes.

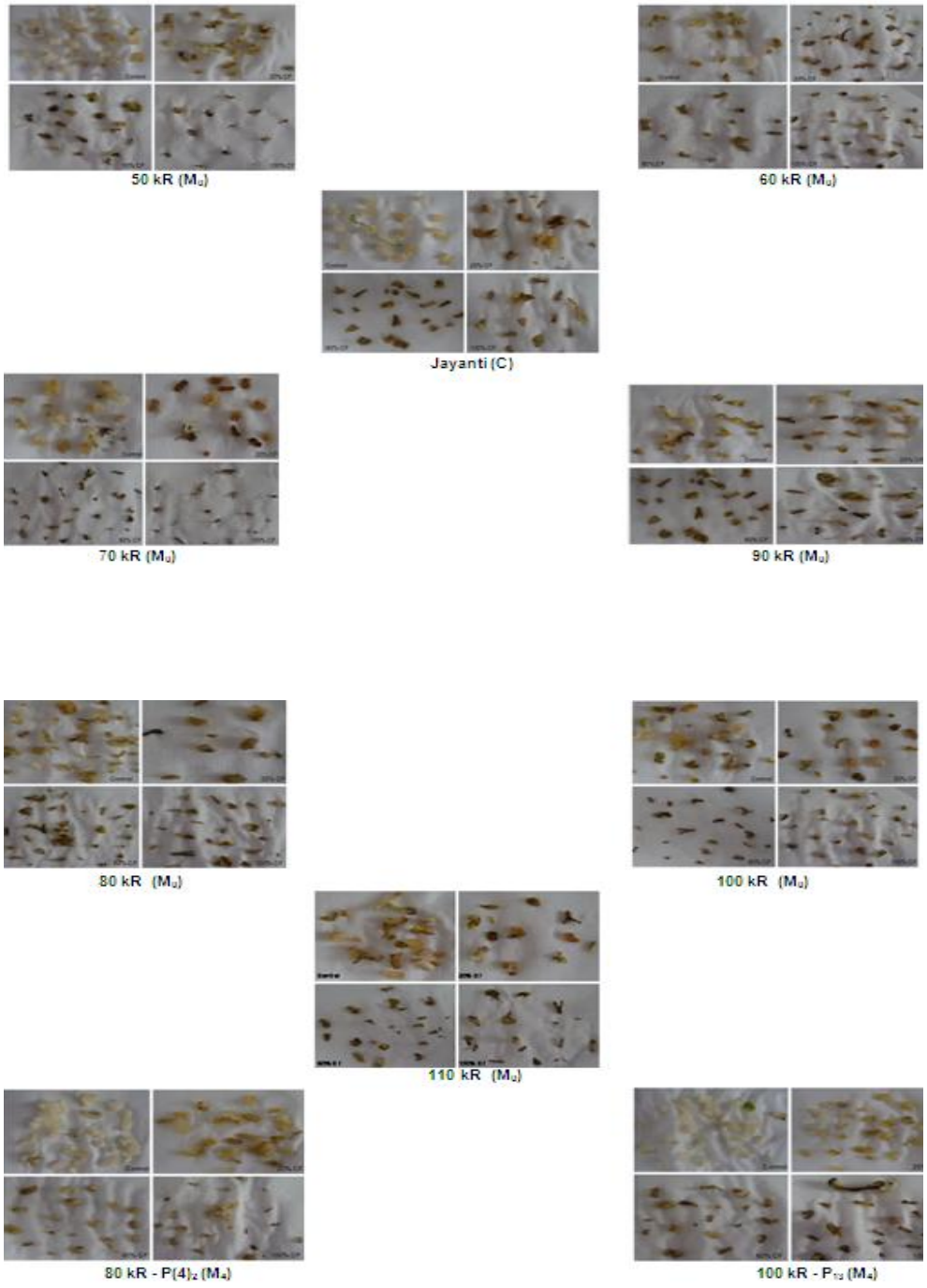

Figure 1. Effect of Cultural Filtrate on different dose-explants through Tissue Culture method in comparison to 'Jayanti'.

Fresh and dry weight of callus

In general, both fresh and dry weights of calli were maximum in 80 kR and 100 kR dose-explants both in M_0 and M_4 generations as compared to 50, 60, 70, 90, and 110 kR dose-explants in M_0 generation. This suggested that the 80 and 100 kR doses exhibited moderate resistance/tolerance to fungal toxin/*Alternaria* blight.

Shoot regeneration

In general, the shoot regeneration potential decreased drastically as the concentration of cultural filtrate

Table 3. Effect of Cultural Filtrate on different dose-explants (in M_0) through Tissue Culture method in comparison to control.

Doses / Character	Control (Jayanti)	50 kR	60 kR	70 kR	80 kR	90 kR	100 kR	110 kR	80 kR* $P_{(4)2}$	100 kR* P_{13}
Without CF										
Callus induction (%)	100	100	96	100	100	94	94	86	100	100
Color of callus	OW	PY	LB	PY	PY	PY	PY	LB	W	W
Fresh wt.(mg)	288	284	173	276	273	180	198	172	581	471
Dry wt.(mg)	68	82	30	33	46	38	63	36	76	80
Shoot response	S	S	S	S	S	NS	S	NS	S	S
20% CF										
Callus induction (%)	100	100	70	85	82	78	88	58	100	100
Color of callus	LB	LB	DB	B	LB	LB	LB	LB	PY	PY
Fresh wt.(mg)	258	257	76	194	248	230	170	93	329	230
Dry wt.(mg)	76	66	40	52	48	72	60	48	124	74
Shoot response	S	S	S	S	S	NS	S	NS	S	S
60% CF										
Callus induction (%)	65	50	50	40	67	43	71	40	75	76
Color of callus	B	B	B	B	B	B	B	B	PY	LB
Fresh wt.(mg)	156	120	38	68	232	160	110	74	234	206
Dry wt.(mg)	24	28	4	24	58	30	24	18	40	36
Shoot response	NS	NS	NS	NS	S	NS	NS	NS	S	S
100% CF										
Callus induction (%)	33	36	32	29	64	42	72	31	63	67
Color of callus	B	B	B	B	B	B	B	B	PY	LB
Fresh wt.(mg)	74	55	40	43	110	52	100	20	207	160
Dry wt.(mg)	14	12	9	14	26	18	14	19	22	28
Shoot response	NS	NS	NS	NS	S	NS	NS	NS	S	S

OW-Off white, PY-pale yellow, W-white, LB-light brown, B-brown and DB-dark brown, S-shoot appears and NS- shoot does not appear, CF-cultural filtrate, *M_4 generation.

increased from 20% onwards. In 20% concentration of cultural filtrate, the shooting response was similar to medium without cultural filtrate. The shoot regeneration was observed in all concentrations of fungal toxin viz., 20, 60, and 100% cultural filtrate in 80 kR dose-explants both in M_0 and M_4 ($P_{(4)2}$) generations as compared to the control Jayanti while 100 kR dose-explants showed regeneration in 20% cultural filtrate only in M_0 generation. On the other hand, 100 kR in M_4 generation (P_{13}) recorded shoot regeneration in all concentrations of fungal toxin viz., 20, 60, and 100% cultural filtrate. Other workers had observed that cell lines resistant to pathogen showed decrease in regeneration of shoots and morphogenetic potential (Singh et al., 1998). It was concluded that, the two mutants viz., $P_{(4)2}$ in 80 kR and P_{13} in 100 kR in M_4 generation had moderate resistance/tolerance to *Alternaria* blight which was confirmed through *in vivo* studies as well. The 80 and 100 kR doses had also exhibited moderate resistance/tolerance in M_0 generation (Table 3).

Thus, this *in vitro* selection can effectively be used to screen genotypes right in M_0 generation itself as 80 kR and 100 kR doses exhibited moderate resistance/tolerance against *Alternaria* blight both in M_0 and M_4 generations. Through *in vivo* selection system, 80 and 100 kR doses exhibited moderate resistance/tolerance against *Alternaria* blight in M_4 generation. Hence, the *in vitro* selection method can be used as an efficient tool for screening mutants dose-wise in previous/initial generations. This method will result in saving time and laborious field work required to screen mutants *in vivo* for a minimum of 3 to 4 generations.

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