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Original Research

In vitro Induced Mutagenesis for Early Blight (*Alternaria solani*) Resistance in Potato (*Solanum tuberosum* L.)

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Abstract

Optimal media compositions were worked out for efficient callus induction and consequent plantlet regeneration using different explants (leaf, node and internode) of potato (*Solanum tuberosum* L.). Among seventeen media compositions used, MS medium supplemented with 3.0 mg/L 2,4-D + 0.25 mg/L BAP exhibited maximum callus induction and proliferation response. Well-proliferated calli were exposed to different treatments of physical (gamma rays 10 to 50 Gy) and chemical (EMS and NaN_2; 1 to 5 mg/L) mutagens and were then screened against partially purified toxin of *Alternaria solani*. Plants regenerated from callus, insensitive to toxin, were supposed to be resistant and were then screened against two different isolates of *A. solani* under field conditions. After two year of field trials four out of 63 *in vitro* screened lines of gamma irradiation; four out of 52 *in vitro* screened lines of sodium azide treated cultures and five out of 59 screened lines of EMS treated cultures were selected according to disease rating scales. Variation was further confirmed using various biochemical and molecular markers.

Keywords: callus induction, disease rating scale, EMS, gamma rays, mutation, plant regeneration, screening, sodium azide, toxin

Introduction

In Pakistan, potato is grown over an area of 0.20 million hectares having annual production of 4.58 million tons and average yield of 22.9 tons/hectare [1], which is far below than other potato growing countries of the world. About 8% of the area for potato cultivation is increasing annually. Moreover, due to susceptibility of disease and abrupt climatic change, the yield potential of varieties under cultivation is deteriorating. Various pathogens can attack potato crops at any stage of growth leading to yield losses (even upto 100% loss). Among them *A. solani* (asexual plant-pathogenic species) causing early blight disease in potato is very common and can spread from one crop to other through its debris or from neighboring infected fields. *A. solani* is polycyclic, i.e. it can infect plants repeatedly in one growing season. This disease

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results in reduced size of tubers leading to loss in crop yield. Use of chemical fungicide is not only expensive but also has social and environmental concerns. This diverts the interest of breeders to introduce resistant genotypes [2]. Climate conditions in Pakistan are generally non-conducive for flowering of potato plants. Nevertheless, this plant develops flowers in some areas, yet hybridization seems impossible due to non-orchestration in flowering. Moreover, conventional plant breeding with desired characters is a slow and time consuming process. This led breeders to explore other possibilities for improvement in desirable trait of genetically stable genotypes without involving sexual cycle. In recent years, *in vitro* induced mutation and screening has emerged as a useful alternative for introducing improved plant genotypes. This has made it possible to grow and multiply mass of cells on a specific medium in culture vial, treat them with mutagen and then screen after exposure to targeted stress. Plants regenerated from such cells can then be shifted for field trials and screening of desired traits. *In vitro* induced mutagenesis offer distinct advantages over conventional breeding being fast screening and propagation under controlled conditions [3]. The successful screening and selection of plants using *in vitro* techniques has been reported against pathogens salinity and frost [4].

Recently, mutation breeding technique has been widely used by plant breeders. Classical breeding approaches and mutagenic radiation methods may induce disease tolerance in many crops. These techniques may enhance agronomic and economically important traits of food crops. The physical and chemical mutagens along with gene editing techniques can induce genetic variability in plants. Chemical mutagens including sodium azide, diethyl sulfate and 1-methyl-1-nitrosourea (MNU) can initiate mutation in plants. The chemicals mostly undergo clastogenesis (chromosome damage) in plants via reactive oxygen species [5]. Badawi et al. [6] revealed that ethyl methane sulphonate (EMS) at concentrations 2 mM/l remarkably induced genetic variability in three cultivars of potato and resultantly nine clones were proved tolerant; four from cv. Atlas, three from cv. Simon and two from cv. Nicola.. After irradiation of plant explants, some biochemical compounds and radicals can be synthesized in tissues. If radiation is administered at a suitable dose and time, positive modifications in resistance, earliness, adaptability and yield of plants may be attained. Gosal et al. [7] observed that application of 20 and 40 Gray (Gy) of gamma radiation dose induced tolerance in *Solanum tuberosum* against fungal disease.

The main objective of this study was to recover early blight resistant plants of potato through *in vitro* induced mutagenesis and screening. For physical mutagenesis γ -radiations were used and for chemical mutagenesis EMS & sodium azide were used. After mutagenic treatment plants were screened *in vitro* against partially purified toxin of *A. solani*. Mutation induced variations can be identified using morphological traits but these morphological variations may also be epigenetic and not heritable. Therefore, biochemical and molecular markers become more reliable to understand the nature of variation/ mutation. To draw correlation between induced mutation and disease resistance some biochemical (quantitative analysis of peroxidases and esterases) and molecular markers for example RAPD (Random Amplified Polymorphic DNA) were studied. Such analysis of mutants helps to understand the nature and proportion of mutation and disease resistance. This study would establish new avenues in the field of plant breeding to explore and introduce new resistant varieties of potato against *A. solani* (early blight) which may be useful to sustain yield of potato.

Material and Methods

For *in vitro* cultures, certified seed tubers of potato (cultivar Asterix) were obtained from Punjab Seed Corporation, Seed farms, Sahiwal. MS medium [8] containing different concentrations and combinations of auxins and cytokinins (pH 5.74±0.03) was used. The optimum temperature standardized for *in vitro* growth was 27ºC±1ºC. For regeneration somatic embryos were first shifted to hormone free MS basal medium and afterwards into MS medium containing various concentrations of hormones (auxins and cytokinins). During hardening, watering was done with Hoagland solution. Potted plants were shifted from greenhouse to screen house and eventually into the field.

Production of Fungal Toxin and Cell Bioassay

Fungus cultures were transformed into spore suspension. Purity of fungus was confirmed using Koch's postulate. Solutions (spore suspension containing 3.1 x 10^5 spores/ mL) were prepared in a twofold dilution series in a duplicate holding buffer with two replicates (50 µL/ well). To isolate toxin from culture filtrate**,** equal volumes of ethyl acetate (EtoAc) were used to make three equal partitions of acidified solution. The fraction of EtoAc were combined, dried over anhydrous sodium sulphate and evaporated to dryness on film evaporator at 40ºC. The aqueous partition was also dried by film evaporator. Finally the holding buffer (100 mL) was used to dissolve residues of both the phases (same volume as for culture filtrate) and used for *in vitro* screening [9].

Induction of Mutation and *in vitro* Screening

After mutation, ethyl methyl sulphonate (EMS) and sodium azide NaN_2 were used. Different concentrations (1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L and 5.0 mg/L) were supplemented in MS media. Seven-week-old calli were cultured on MS medium comprising chemical mutagen (EMS or NaN_2) for five days, then shifted as appropriate on fresh MS medium for further proliferation and plant regeneration [10].

In vitro Screening of Resistant Plants

After mutagenic treatment, the calli were screened against with partially purified toxin (*A. solani*). For *in vitro* screening, toxin (63 units/mL toxicity) was added (0.05% to 0.5%) in MS medium supporting callus proliferation and growth. Field screening was done according to disease rating scales [11]. The allocated scores obtained for specific plant acquired during the 4 scoring durations during one growing season were then employed to compute the area under the disease progress curve (AUDPC) for each plant [12]. AUDPC was analyzed with the help of following equation:

$$AUDPC = \Sigma i=1 n-1[(ti+1 - ti)\ (yi + yi+1)/2]$$

Value of AUDPC was used to evaluate the relative AUDPC (RAUDPC) of each plant.

Biochemical Characterization of Control and Selected Resistant Lines

Fresh leaves of plant were dried till constant weight (70ºC for 48 h in oven) to calculate dry biomass.

Estimation of Total Soluble Protein Contents and Enzymatic Activity

The soluble protein contents were determined using Bradford, [13] assay with bovine serum albumin as the standard curve. Carrot leaves of 1 g fresh weight were ground with liquid nitrogen and homogenized in 10 mL of 0.1 M potassium phosphate buffer (pH 7.0). The supernatant obtained following 10 min centrifugation at 12,000 rpm was used for the estimation of the enzymatic activity.

Phenylalanine Ammonium Lyase

Specific activity of phenylalanine ammonium lyase was determined by Zucker [14] methods with some modifications. For this purpose, 0.3 mL plant extract was mixed with borate buffer (1.35 mL of 200 µM) and phenylalanine (1.35 mL of 30 mM), incubated at 40ºC for 1 h. To terminate the reaction, 0.2 mL of 5 N HCl was added. The volume was increased up to 4 mL with distilled water. The absorbance was noted at 270 nm.

PolyPhenol Oxidase

For PPO activity, the method was selected from the Worthington Enzyme Manual by Decker methodology [15]. In this method, 0.1 mL enzyme extract, 1.0 mL of 0.001 M tyrosine, 1.0 mL phosphate buffer and 0.9 mL water were mixed. After 10-15 min, absorbance was recorded at 280 nm with the help of a spectrophotometer, which was later used to calculate the specific activity of PPO.

Peroxidase

Method proposed by Abeles and Biles, [16] was used to calculate the specific activity of peroxidases. 0.2 mL plant extract, 2.5 mL phosphate buffer, 0.2 mL (1%) guaiacol and 0.1 mL (0.3%) H_2O_2 were mixed. Absorbance was recorded at 470 nm using a spectrophotometer which was used to determine the specific activity of peroxidases.

Determination of Phenolic Content

A homogenous mixture was prepared using liquid N_2 using 30% methanolic extract. Gallic acid was used as an internal standard and calibrations were carried out spectrophotometrically [17].

Estimation of Esterases

Substrate preparation: α-nephthyl acetate (558.3 mg) was dissolved in acetone (100 mL). One mL from this solution was added in 99.0 mL (0.2 M) phosphate buffer (pH 7.0), incubated at 25ºC for 30 minutes. Then freshly prepared 0.1 mL stain solution (7.0 mL 5% SDS and 3.0 mL water containing 30.0 mg of fast blue R.R salt) was added, incubated for 15 minutes and absorbance was recorded at 605 nm [18].

Random Amplified Polymorphic DNA (RAPD) Analysis

DNA was extracted from fresh leaves of control and selected mutants by CTAB method from Doyle and Doyle, [19].

Statistical Analysis

The data comprising mean values of 5 biological replications were subjected to one-way analysis of variance and subsequently to Duncan's Multiple range test.

Results and Discussion

Callus Induction and Plant Regeneration

Among different explants used in present study (i.e. leaf, node and internode) leaf depicted highest callus induction and proliferation response when cultured on MS medium containing 3.0 mg/L 2,4-D in combination with 0.25 mg/L BAP(CM_6), (i.e., 98% callus induction from leaf and nodal explants and 96% from intermodal explants). By increase or decrease in the concentration of either 2,4-D or BAP callus induction and proliferation response of all explants was decreased. All other concentrations of 2,4-D either alone or in combination with BAP, kinetin or NAA failed to show better results (Table 1). In complete darkness, frequency of callus formation and proliferation was found to be higher. Mostly nodular, compact and

morphogenic callus was obtained in darkness at 27±1ºC. Well proliferated morphogenic calli obtained from fifth subculture after 12 week of inoculation were shifted for organ induction. Best results for organogenesis were obtained when MS medium was supplemented with 1.0 mg/L BAP i.e. shoot induction response was 68%, 62% and 52% by callus obtained from leaf, node and internode, respectively. All other combinations of BAP or Kinetin failed to show good organogenic response. Among different explants used, callus derived from leaf explants exhibited best results for organ (shoot and root) induction, followed by node and internode, respectively (Table 2).

Callus proliferates logarithmically via initial slow division of cells followed by rapid cell division (including active synthesis of DNA, RNA and proteins) leading to differentiation into regenerated plants. 2.4-D for callus induction has proven its vitality for almost all types of plants [20]. For explants which failed to show good callus induction response in the presence of 2, 4-D, addition of small amounts of cytokinin proved to be effective [21-22]. Specific receptor sites for auxins are present on cells which probably are some auxin binding protein [23] that activates the plasma membrane H^+ ATPase. The ability of protons (H^+) to cause loosening of cell wall is mediated by class f proteins called expansions which also break the hydrogen bonds of polysaccharides components of cell wall [24]. These intercellular calcium ions are second messengers for early action of auxins. Calcium ions alone or with calcium binding proteins (calmodulin) activates cascades of protein kinase leading to the activation of other proteins and transcription factors which interact with auxin-responsive elements that regulate their gene expression affecting cell cycle to stimulate cell division [25]. The length of callus induction phase depends mainly on the physiological status of the explant as well as cultural conditions. Nodal and internodal explants secrete phenolic compounds which causes the oxidation of sucrose resulting in depletion of carbon source in medium that lead to browning and necrosis of cultures. Explant consisting of meristematic regions proliferated quickly because cells divide immediately without de-differentiation, while explants taken from differentiated cells must go to de-differentiation before cell division (Table 1). In the present study it was also noticed that when cuts were marked with scalpel on the surface and margins of leaf explants it enhances callus induction response. This may be due to the reason that making cuts on leaves trigger the totipotency of affected cells that enhance their response to exogenous growth hormones. Daffalla et al. [26] further added that making cuts at the margins of leaf explants enhanced their potential to form callus.

Organogenesis in dicots is a common regeneration feature because explants as well as *in vitro* conditions are simple and robust but its pathway is not strictly the same in all plant species. Possible reason may

Table 1. Effect of different hormones on callus induction and proliferation.

S.No.	Medium	Conc. (mg/l)	Callus induction response					
			Leaf		Node		Internode	
			Test tubes	Rate of callus Induction (%)	Test tubes	Rate of callus Induction (%)	Test tubes	Rate of callus Induction (%)
CM_1	MS Basal Control)	0.0	00	00	00	00	00	00
CM_2	MS+2,4-D	1.0	3.2 ± 0.521^{f}	32	2.6 ± 0.219^{f}	26	2 ± 0.48^{f}	20
CM_3		2.0	5.2 ± 0.657^{e}	52	5.4 ± 0.456^{e}	54	4.8 ± 0.334^{e}	48
CM_4		3.0	8.6 ± 0.357^{abc}	86	7.8 ± 0.521^{bc}	78	7.4 ± 0.726^{bc}	74
CM_5		4.0	7.8 ± 0.334^{bcd}	78	7 ± 0.565^{cd}	70	7 ± 0.748^{cd}	70
CM_6	MS+2,4-D+ BAP	3.0+0.25	9.8 ± 0.178^{a}	98	9.8 ± 0.178^{a}	98	9.6 ± 0.219^{a}	96
CM_7		3.0+0.50	9.4 ± 0.357^{a}	94	8.8 ± 0.438^{ab}	88	8.6 ± 0.456^{ab}	86
CM_8		4.0+0.25	8.6 ± 0.456^{abc}	86	8.2 ± 0.521^{bc}	82	7.4 ± 0.334^{bc}	74
CM_9		4.0+0.5	7.6 ± 0.606^{bcd}	76	7 ± 0.56^{cd}	70	7 ± 0.282^{cd}	70
CM_{10}	MS+2,4-D + KIN	3.0+0.25	8.8 ± 0.438^{ab}	88	8 ± 0.4^{bc}	80	8.2 ± 0.334^{bc}	82
CM_{11}		3.0+0.50	8 ± 0.282^{bcd}	80	8 ± 0.489^{bc}	80	7.8 ± 0.334^{bc}	78
CM_{12}		3.0+1.00	7.4 ± 0.357^{cd}	74	7.2 ± 0.521^{cd}	72	7.2 ± 0.456^{c}	72
CM_{13}		4.0+0.5	6.8 ± 0.334^{d}	68	6.2 ± 0.178^{de}	62	5.8 ± 0.438^{de}	58
CM_{14}	MS+ NAA + BAP	2.0+0.25	5 ± 0.632^{e}	50	5.4 ± 0.219^{e}	54	5.2 ± 0.178^{e}	52
CM_{15}		2.0+0.50	5.2 ± 0.593^{e}	52	5.4 ± 0.456^{e}	54	5 ± 0.282^{e}	50
CM_{16}		3.0+0.50	7.8 ± 0.334^{bcd}	78	7.8 ± 0.334^{bc}	78	7.6 ± 0.357^{bc}	76
CM_{17}		3.0+1.00	7.4 ± 0.219^{cd}	74	7.4 ± 0.219^{cd}	74	7.0 ± 0.489^{cd}	70
	LSD		1.39		1.34		1.27	

Test Tubes culture = 10

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test

Table 2. Organogenesis from morphogenic callus.

Treatment No.	Media	Conc. (mg/l)	Organogenic Response					
			Leaf		Node		Internode	
			Test tubes	Rate of shoot induction (%)	Test tubes	Rate of shoot induction (%)	Test tubes	Rate of shoot induction (%)
MC_1	MS Basal		4±0.04defg	40	3.8±0.521^{c}	38	2.6±0.537cde	26
MC_2	MS + 2,4-D + BAP	0.25+ 0.25	2.2±0.005^{h}	22	1.8±0.178	18	1.2±0.178^{f}	12
MC_3		0.25 + 0.5	2.6±0.219^{gh}	26	2.2±0.438	22	1.4±0.219^{ef}	14
MC_4		0.5 + 1.0	3.8±-.438^{efg}	38	3.2±0.593	32	2.2±0.334^{cde}	22
MC_5		0.5 + 2.0	3.6±0.357^{fgh}	36	3.2±0.521	32	2±0.282d^{ef}	20
MC_6	MS + BAP	0.5	5.2±0.334^{bcde}	52	5.2±0.17	52	4.4±0.357^{ab}	44
MC_7		1.0	6.8±0.438^{a}	68	6.2±0.52	62	5.2±0.178^{a}	52
MC_8		1.5	6.4±0.456^{ab}	64	6±0.282^{a}	60	4.8±0.334^{a}	48
MC_9		2.0	4±0.282^{defg}	40	3.4±0.21	34	2.2±0.438^{cde}	22
MC_{10}	MS + Kinetin	0.5	4.6±0.357^{cdef}	46	4±0.489bc	40	3.4±0.357^{bc}	34
MC_{11}		1.0	5.6±0.219^{abc}	56	3.8±0.438	38	2.2±0.521^{cde}	22
MC_{12}		1.5	5.2±0.179^{bcde}	52	4±0.565bc	40	2±0.489^{def}	20
MC_{13}		2.0	2.8±0.438^{gh}	28	3.2±0.17	32	1.8±0.334^{ef}	18
MC_{14}	MS + BAP + Kinetin	0.25 + 0.25	2.8±0.593^{gh}	28	3.2±0.334	32	1.8±0.178^{ef}	18
MC_{15}		0.5 + 0.25	5.4±0.456^{abcd}	54	5.4±0.606	54	3.2±0.334^{bcd}	32
MC_{16}		1.0 + 0.50	6.2±0.995^{ab}	62	5.6±0.219	56	3.2±0.593^{bcd}	32
MC_{17}		2.0 + 0.50	3.6±0.726^{fgh}	36	4.2±0.438	42	2.6±0.669^{cde}	26
LSD			1.49		1.32		1.22	

No of Test tubes cultured = 10 Age of culture = 10 week

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test

be that the elements behind totipotency that provide regeneration ability are yet not well known. All cells of callus do not undergo organogenesis as there exists cellular heterogeneity, which actually is heterogeneity in gene expression. Organogenesis on hormone free medium may be due to the presence of meristematic cells in explants [27-28]. For other cells, addition of cytokinins may trigger the ability of certain cells to grow in organized fashion resulting in the conversion of parenchyma cells to meristemoids (centre of meristematic activity) leading to organ formation. Role of cytokinins for shoots induction from callus suggest the development of shoot apical meristem (SAM) which may be by the regulation of gene expression. A group of genes called knotted1 (kn1) homeobox genes, are exclusively reported in SAM [29]. According to Kitagawa and Jackson, [30] these genes are under the control of a heat shock promoter which is correlated to elevated levels of cytokinins. Therefore, cytokinins are believed to have a role in regulating gene expression for development of SAM. In the present study higher organogenic response from explants of newly formed leaves (Table 2) and node may be due to the reason that these explants contain pre-existing buds that give it greater meristematic nature [31]. The loss of organogenic potential with age of culture in present study may be due to increase of chromosomal disorder.

Effect of Gamma Rays Exposure on Proliferation and Morphogenesis of Callus

Table 3 depicts the effect of gamma rays exposure on callus proliferation and plant regeneration. Data shows that in the control 1.66 gm callus was formed after 15 week. On regeneration, 7.2 plants were obtained from this callus. After 10 Gy and 20 Gy exposures to gamma rays, a stimulatory effect was noticed i.e. after 10 Gy and 20 Gy exposures, the weight of proliferated calli was 1.71 gm and 1.79 gm which was 3.01% and 7.83% more as compared to control, respectively. When these irradiated calli were shifted to the regeneration medium, the rate of plant regeneration was 5.55% and 13.89% more over control with an average of 1.4 and 3.8 morphological variants, respectively. When exposure time was increased, callus proliferation as well as regeneration response was gradually decreased. At 30Gy exposure, callus proliferation was 1.52 g which on regeneration produced 6.6 plants with 2-3 visible variants (8.43% less callus proliferation and 8.33% less regenerated plants as compared their respective controls). At high exposure of γ- rays, poor callus proliferation and regeneration was recorded. At 40 and 50Gy exposures the callus proliferation was 1.39 gm and 1.18 gm which was 16.26% and 28.92% less than control. When shifted to the regeneration medium, 4.8 and 3.8 plants were regenerated which was 33.33% and 47.22% decrease as compared to control with

Table 3. Effect of gamma rays exposure on callus proliferation and plant regeneration.

γ-rays exposure (Gy)	Culture age (week)	Callus		Plants regeneration		Morphological variations				
		Proliferation (gm) (After 3 week exposure)	% Increase / decrease over control	Number of plants / culture vial (After 5 week exposure)	%Increase decrease over control	Viridis	Albino	V	T	S
Control	15	1.66±0.021^{b}	---	7.2±0.334^{ab}	---	1.2±0.178^{ab}	00	00	00^{b}	0.6±0.219^{a}
10	15	1.71±0.047^{ab}	+3.01	7.6±0.219^{ab}	+5.55	1.4±0.357^{ab}	00	00	00^{b}	0
20	15	1.79±0.021^{a}	+7.83	8.2±0.334^{a}	+13.89	1.8±0.334^{a}	0.8±0.178^{b}	0.6±0.357^{a}	00^{b}	0.6±0.219^{a}
30	15	1.52±0.017^{c}	-8.43	6.6±0.456^{b}	-8.33	0.8±0.178^{b}	1.0±0.282^{ab}	00	0.6±0.219^{a}	00
40	15	1.39±0.009^{d}	-16.26	4.8±0.521^{c}	-33.33	1.0±0.282^{ab}	1.8±0.334^{ab}	00±	0^{b}	0
50	15	1.18±0.015^{e}	-28.92	3.8±0.178^{d}	-47.22	0.8±0.178^{b}	2.0±0.4^{a}	1.2±0.334^{a}	0^{b}	0
LSD		0.08		1.09		0.85	1.03	1.26	0.476	0.79

Different alphabets on mean in one column denotes level of significance at P = 0.05 according to DNMR test. T = Thin leaves V = Vitrified S = Small Plants

an average of 3 and 4 morphological variants, respectively (Table 3).

Induced mutation has played a significant role to improve desirable genetic traits especially for crops like potato having complex and narrow genetic makeup. For physical mutagenesis γ rays are commonly used to get a large number of mutants in the shortest possible time [32]. Low exposures of γ rays may stimulate cell division and growth by altering signaling networks of hormones or by enhancing the antioxidative capacity of plant cells. High exposures of radiation arrest cell cycle at G2/M phase during somatic cell division [33]. Dividing cells are more sensitive to gamma rays as compared to stationary cells therefore in present study damages of irradiations were greater to embryogenic cells than callus which ultimately regenerated less number of plants (Table 3). Radiations mainly interact with water molecules in biological systems (55-80%) leading to the formation of hydrogen peroxide, hydroxyl and the superoxide radicals [34]. These oxidized polyunsaturated fatty acids are present in cell membranes and enter the nucleus causing oxidation of purines, pyrimidines and sugar molecules that split double bonds present in DNA affecting genetic code. In DNA, free radicals due to radiolysis of water cause radiation-induced DNA damages [35]. Ionizing radiations may disrupt double helix by strand breaks in DNA [36]; double strand break [37] or crosslinking of DNA to proteins or to itself etc. such multiple damages of radiations leads to mutation.

Induction of Mutations Using Ethyl Methyl Sulphonate (EMS)

After 1.0 mg/L and 2.0 mg/L treatment of EMS, weight of proliferated calli was 1.41 g and 1.28 g which was 7.84% and 16.33% less as compared to control. Number of regenerated plants was 20% and 25.71% less, respectively, as compared to the number of plants regenerated from control cultures with only 3 morphological variants. The rate of callus proliferation and plant regeneration was gradually decreased with increase in the concentration of EMS treatment. After treatment of 3 mg/L EMS, weight of proliferated calli was 1.13g which on regeneration produced 4.2 plants with only 02 morphological variants i.e. a decrease of 26.14% in callus proliferation and 40% in plant regeneration when compared with control. After 4 mg/L and 5 mg/L treatment of EMS, weight of proliferated calli was 1.04g and 0.88g, respectively, (i.e. 32.03 % and 42.48% less as compared to control) which on regeneration produced 3.0 and 2.2 plants (7.14% and 68.57% decrease in regenerated plants as compared to control) with 1.6 and 1 morphological variants, respectively (Table 4).

Chemical mutagens induce a higher rate of gene mutations. Among them, azides and alkylating agents are most extensively used. EMS alkylates guanine base, which pairs with thyamine instead of cytosine leading to Guanine/Cytosine-to-Adenine/Thymine transitions

that can produce a high frequency of nucleotide substitutions in many organisms [38]. Data presented in Table 4 exhibits that weight of proliferated callus decreased gradually by increasing EMS treatment. Similarly regeneration potential of callus decreased at higher concentration of EMS This is because higher concentration of EMS causes necrosis of callus. At 5.0 mg/L concentration of EMS plant regeneration was 68.57% less as compared to control (Table 4). Among different type of morphological variations mostly chlorophyll variants (viridis and albino) were more common. Moon et al., [39] also observed more chlorophyll variants after treatment with EMS in potatoes. Sodium azide causes point mutagenesis of plants and has well been evaluated in callus cultures of *Phaseolus vulgaris* L. [40]. In the present study, the rate of callus proliferation and plant regeneration decreased by increasing the concentration of sodium azide (Table 5).

Sodium Azide (NaN_2) Induced Mutagenesis

Table 5 depicts that incorporation of NaN_2 into MS medium adversely affected the rate of callus proliferation and plant regeneration. When the concentration of NaN_2 was increased from 1 mg/L to 5 mg/L, the rate of callus proliferation was decreased to 1.52 g, 1.46 g, 1.33 g, 1.21g and 1.08 g at 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L and 5.0 mg/L NaN_2 which was 2.56%, 6.41%, 14.74%, 22.44% and 30.76% less as compared to control, respectively. It is also obvious from table that number of plants regenerated from sodium azide treated calli were 6.8, 6.2, 4.6, 3.4, 2.6 at 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L and 5.0 mg/L NaN_2, respectively. Maximum morphological variants were regenerated at 3 mg/L and 4 mg/L NaN_2 (Table 5).

After treatment with sodium azide, chlorophyll variants were predominant. Sodium azide is an S stage mutagen that acts through organic metabolite (pro-mutagens) that has been identified as azidoalanine [41]. It usually induces base substitutions (mostly induced Guanine: Cytosine → Adanine: Thyamine transitions at the 2nd codon position) and also produce DNA single strand breaks. Kolar et al. [42] reported chromosomal aberrations by using sodium azide, however, reciprocal translocations and mitotic chromosomal aberrations have also been reported by sodium azide [43]. It has also been reported as an electron transport blocker, inhibitor of the respiratory chain and its immediate effect on the meristematic region is the dropping off its progression velocity in the cell cycle [44], its exact mechanism of action as a chemical mutagen is still not clear. In the present study, more variants were obtained using radiation as compared to chemical mutagens. This may be due to the reason that chemical mutagens may influence cells only direct contact with the mutagen or their concentration used in experiments was low while radiations likely to be more effective due to their deep penetration into multicellular tissues.

Table 4. Effect of different treatments of EMS on callus proliferation and plant regeneration.

Conc. Of EMS used (mg/l)	Age of culture (week)	Callus		Regenerated plants		Morphological variations observed			
		Proliferation (gm)	% Increase/decrease over control	Number of plants	%Increase/decrease over control	Viridis	Albino	V	T
Control	15	1.53±0.011	---	7±0.632^{a}	---	00c	00c	00^{b}	00^{b}
1.0	15	1.41±0.026	-7.84	5.6±0.357^{b}	-20	00^{b}	1.8±0.334^{a}	00^{b}	1±0.282^{a}
2.0	15	1.28±0.019	-16.33	5.2±0.178^{bc}	-25.71	1.0±0.521^{a}	1.2±0.178^{b}	00^{b}	00^{b}
3.0	15	1.13±0.006	-26.14	4.2±0.334^{cd}	-40	0.8±0.334^{b}	0.5±0.178^{b}	1.0±0.4^{a}	00^{b}
4.0	15	1.04±0.023	-32.03	3.0±0.282^{de}	-57.14	0.6±0.219^{b}	00c	1.0±0.4^{a}	00^{b}
5.0	15	0.88±0.021	-42.48	2.2±0.334^{e}	-68.57	00^{c}	00c	0.8±0.178^{a}	00^{b}
LSD		0.063		1.238		0.875	0.558	0.692	0.376

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test.

Table 5. Effect of different treatments of Sodium azide on callus proliferation and plant regeneration.

Conc. Of NaN_2 used (mg/l)	Age of culture (week)	Callus		Regenerated plants		Morphological variations observed			
		Proliferation (gm)	% Increase/decrease over control	Number of plants	% Increase/decrease over control	Viridis	Albino	V	T
Control	15	1.56±0.032^{a}	---	7.2±0.438^{a}	---	00	00^{b}	00^{b}	00^{b}
1.0	15	1.52±0.017^{ab}	-2.56	6.8±0.338^{a}	-05.55	0.8±0.178^{a}	1.2±0.334^{a}	00^{b}	00^{b}
2.0	15	1.46±0.021^{b}	-6.41	6.2±0.179^{a}	-13.89	1.0^{a}	1.2 ±0.178^{a}	0.6±0.219^{ab}	0.8±0.438^{ab}
3.0	15	1.33±0.011^{c}	-14.74	4.6±0.537^{b}	-36.11	1.4±0.357^{a}	1.4±0.219^{a}	0.8±0.334^{a}	01±0.4^{a}
4.0	15	1.21±0.01^{d}	-22.44	3.4±0.456^{bc}	-52.78	00	1.0±0.282^{a}	0.8±0.178^{a}	01±0.282^{a}
5.0	15	1.08±0.022^{e}	-30.76	2.6±0.219^{c}	-63.89	1.4±0.219^{a}	0.8±0.178^{a}	00^{b}	00^{b}
LSD		0.067		1.249		0.607	0.734	0.583	0.875

Means followed by different letters in the same column differ significantly at P = 0.05 according to Duncan's new multiple range test. T = Thin leaves V = Vitrified S = Small Plants

Field Screening and Study of Morphological Characters

Area Under Disease Progressive Curve

Minimum AUDPC for Gamma, EMS and SA was calculated as 45, 82 and 45, respectively. Mean and standard deviation of AUDPC was observed 235.83±20, 204.41±16 and 224.86±19.09 for Gamma, EMS and SA, respectively (Fig. 1). Although some extreme values were detected in EMS but overall inter-quartile range (50% of the observations) was 135-318 for Gamma, 127-217 for EMS and 127-283 for SA (Fig. 2). Using completely randomized design (CRD) under five replications, one-way analysis of variance for seven morphological traits explained the variation due to mutation in fourteen representative plants. Shoot length (cm) was affected up to 38 percent as compared to the control and the effect of physical as well as chemical mutation on shoot length was significant (p-value<0.01) with 3.57% coefficient of variation of the model. As a result of the least significant difference test ($LSD_{0.05}$) the critical value of 2.62 for shoot length reveals that there are four significant groups within which the means are not significantly different from each other.

Critical t-value for 0.05 alpha and 56 degree of freedom was 2.003 for all of the seven morphological traits. Effect of mutation on shoots per plant (SPP) and nodes per shoots (NPS) was significant at p-value <0.0001 and <0.01, respectively. In SPP and NPS, 51 and 39% variation was observed due to the mutation effect in SPP and NPS with 10.45 and 9.58% coefficients of variation of this univariate model for the traits SPP and NPS, respectively. Critical value for comparison ($LSD_{0.05}$) for NPS and SPP was 1.87 and 1.14 which revealed 3 and 5 significant groups, respectively, where the means within the groups were non-significant. Tuber parameters such as tubers per plant (TPP), tuber size (TS), tuber weight (TW) and eyes per tuber (EPT) were also significantly (p-value <0.001) affected by the mutation and causes 45, 41, 42 and 50% variation with 12, 11.5, 6 and 13% coefficient of variation of the CRD model, respectively. As a result of the LSD test, only two groups were observed in TPP, TS and TW with 1.48, 1.43 and 10.57 critical values of comparison, respectively. Three groups were revealed in eyes per tuber (EPT) with critical value of 1.52 for comparison at alpha 0.05.

Callus or somatic embryos after treatment with mutagens can be screened *in vitro* using toxin treatments, usually cells resist/tolerant to toxins when regenerated into plants can further be multiplied *in vitro*. Pathogenicity of the fungus in present study was confirmed by Koch's postulate. The toxin was produced from liquid culture in PDA medium. It was sieved (0.45 micron) and partially purified before use [45]. It is believed that tissues surviving after mutagenic treatment when screened against toxins or culture filtrate, results in a small proportion of viable cells which are believed

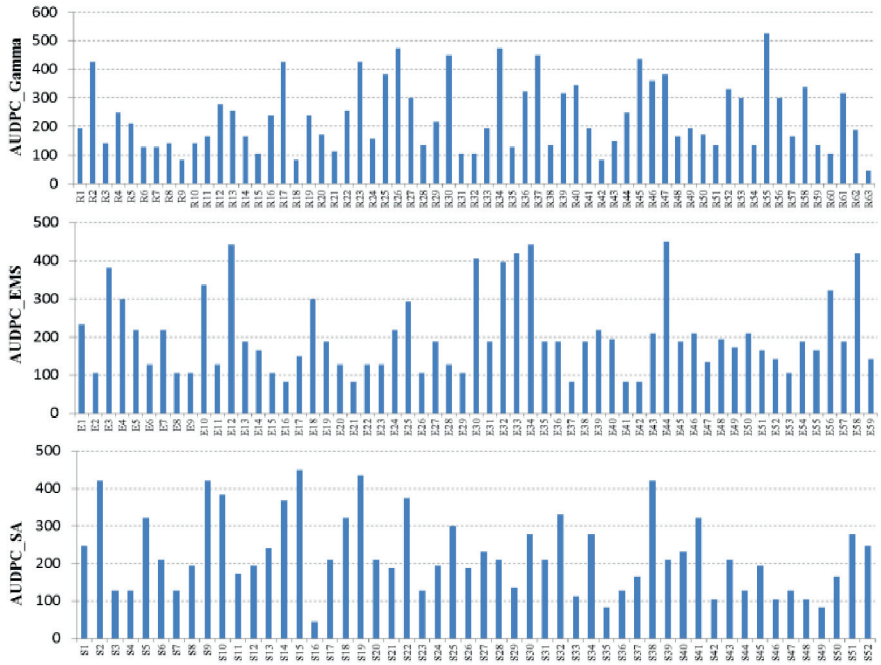

Fig. 1. Area under disease progress curve (AUDPC).

to regenerate into resistant genotypes. Several studies are available reflecting the use of fungal culture filtrate against callus in tissue culture or protoplasts [46] to obtain resistant plants. *Alternaria* species (both host-specific or non-host specific) are well known for phyto-toxin production. *Alternaria* mycotoxins have commonly been reported in vegetables and fruits. Potential virulence factors and genes related to pathogenesis of *Alternaria* toxins have recently been noticed by Meena et al., [47]. In Potato for *in vitro* screening using *A. solani* only few reports are available, however reports are available for screening Potato and Tomato under field conditions using toxins of *A. solani*. Present study, reflects the possibility of *in vitro* screening of Potato against *A. solani*. Similarly Mirkarimi et al. [48], used culture filtrate of *A. solani* to screen against early blight in potato.

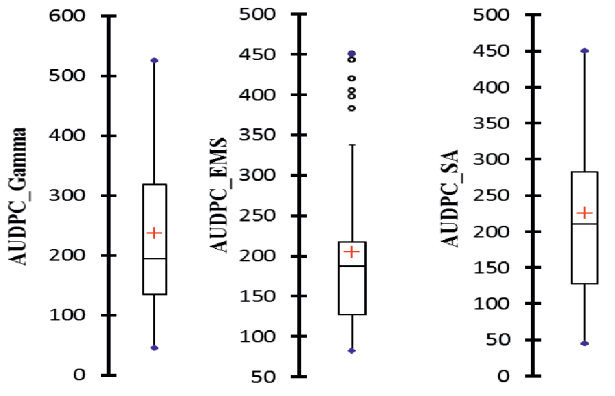

Fig. 2. Descriptive graph of Area under Disease Progress Curve (AUDPCs).

Genetic stability due to *in vitro* induced mutation can be confirmed under field conditions. Field trials for disease resistance require screening of mutants against pathogens. Regenerated plants with different levels of resistance and susceptibility reflect the existence of genetic variability. Careful examination and interrelations of morphological attributes have great contributions in potato breeding because these allow breeders to identify promising lines efficiently and effectively. Xu et al. [49], reported that combined evaluation of characters like number of leaves/plant, number of tubers/plant and average weight of tubers exhibit high degree of positive association with yield of Potato crop. In Potato, for early generations, selection is based on the visual inspection of better appearance of tubers in combination with other agronomic traits. The selected lines are compared with control for parameters like tuber weight, tuber size, number of eyes/tuber and eye depth. Then advanced lines are selected gradually through several selection cycles. These morphological parameters have been used frequently for successful development of the number of cultivars [50]. Al-Safadi et al. [51] studied the effect of gamma radiations for microtuber production in potato and reported that treatment of explant with 2.5 Gy of gamma radiations significantly increased the number of microtubers in potato. Afrasiab and Iqbal, [52] reported a considerable variation in number of shoots per plant, number of tubers per plant, tuber size and tuber weight as a result of gamma radiations. In present study, mutants screened after chemical mutagenesis also exhibited an increase in agronomic traits over control (Table 6). Similarly Alsoudan et al. [53] identified morphological variants in Barley as a result of treatment of sodium azide.

Table 6. Analysis of variance of seven morphological traits with complete randomized design (CRD) using five replicates for 14 varieties.

Trait	Mean squares			Grand	
	Variety	Error	F	Mean	CV%
SL	11.3221	4.28700	2.64**	57.92	3.57
SPP	3.6615	0.80714	4.54***	8.6	10.45
NPS	6.2297	2.17143	2.8**	15.39	9.58
TPP	4.7956	1.35714	3.53***	9.77	11.92
TS	3.8209	1.27143	3**	9.74	11.57
TW	222.8747	69.57857	3.2**	140.1	5.96
EPT	6.2209	1.43571	4.33***	11.56	10.37

Degree of freedom: df-variety = 13, df-error = 56; SL: Average shoot length;SPP: Average shoots per plant; NPS: Average nodes per shoot; TPP: Tubers per plant; TS: Average tuber size; TW: Average tuber weight; EPT: Eyes per tuber; *, **, ***: p-value <0.05, <0.01, <0.001

Heat map of six biochemical parameters in 14 selected plants shows minimum (Min.), median (Med.) and maximum (Max.) values (Fig. 3). Control and S2 selected resistant lines have minimum while R3 and E3 have maximum phenolic contents. Mutant E3 also has maximum peroxidases but minimum esterases. Total soluble proteins were maximum in mutant E1 and minimum in control and mutant S4. The parameter PAL was maximum in mutant E5 and minimum in mutant E2 and the parameter PPO was maximum in R1, R4, S3 and minimum in control, E1 and E5. Based on six biochemical parameters, using similarity obtained from Pearson correlation coefficients, Agglomerative hierarchical clustering (AHC) of 14 representative plants was performed with unweighted pair-group average (UPGA) method under automatic truncation. This AHC method classified 14 plants in three subgroups. Seven plants including control, R2, R3, E2, E3, S1 and S2 were placed in class 1 (Figs 3 and 4). In class 2, there were four plants R1, R4, S3 and S4 while three plants (E1, 34, E5) were classified in class 3. Threshold similarity for these three classes was set at >0.996. In this clustering within and between class variance was 36.29 and 63.71%, respectively. Principal component analysis (PCA) was performed using six biochemical parameters as variables and fourteen plant types as observations. Three classes obtained from AHC analysis were used as supplementary information in the PCA. Components along the x-axis (PC1: eigenvalue 1.94) explained 32% of the overall variation and the component along the y-axis (PC2: eigenvalue 1.378) explained 23% variation.

As a whole the analysis captured 55% variation. Phenolic contents (TPC), peroxidases, esterases and PAL were in linear combination with PC1 and contributed about 95% to the variation explained by PC1. Soluble proteins (TSP) and PPO were linear with PC2 and contributed about 77% to the variation explained by PC2. Plants in class-1 showed spread along PC1 under the influence of a single factor being represented by PAL, esterases, peroxidases and TPC. The Plants in class-3 were positively associated with TSP, PAL, TPC along PC2 and the plants in class-2 were negatively associated with PC2 under the positive influence of PPO and peroxidases.

Mutagenesis of well proliferating calli may bring variations of different magnitude either stable or unstable. Among different gene products, the level of specific enzymes or proteins can be determined to assess variation at genetic level [54]. These genetic products reflect genetic differences. This study was designed to find out correlation between biochemical processes and disease resistance.

Stress caused by mutagenic treatment (either physical or chemical) may also cause a change in metabolism of proteins and some new proteins may also be synthesized which may have effect against pathogens by recognizing specific effector molecules formed during infection. During infection, various pathogen resistant (R) proteins such as chitinases and β-1, 3-glucanases are produced in uninfected plant parts and lead to systemic acquired resistance thus protecting plants from further infection. These R-proteins also cause hydrolysis of the fungal cell wall that ultimately leads to lysis of fungal cells. In addition, they are also responsible for the development of oligosaccharide elicitors that reduce the production of other R proteins or low molecular weight antifungal compounds, such as phytoalexins [55]. These proteins are considered as on/off switches to regulate resistance in plants through protein accumulation or allosterism. A potential role of proteins on pathogens recognized indirectly may be that escaping recognition requires loss of effector function that ultimately leads to loss of pathogenicity. These R proteins may act as primary receptors for pathogen effector proteins [56]. Different R proteins function either by specific localization within the host cell or by phosphorylation. Defense response has also been reported in the identification of pathogenesis related proteins from a broad range of host species. Analysis of the protein sequences (of plant defense) of a wide range of monocot and dicot

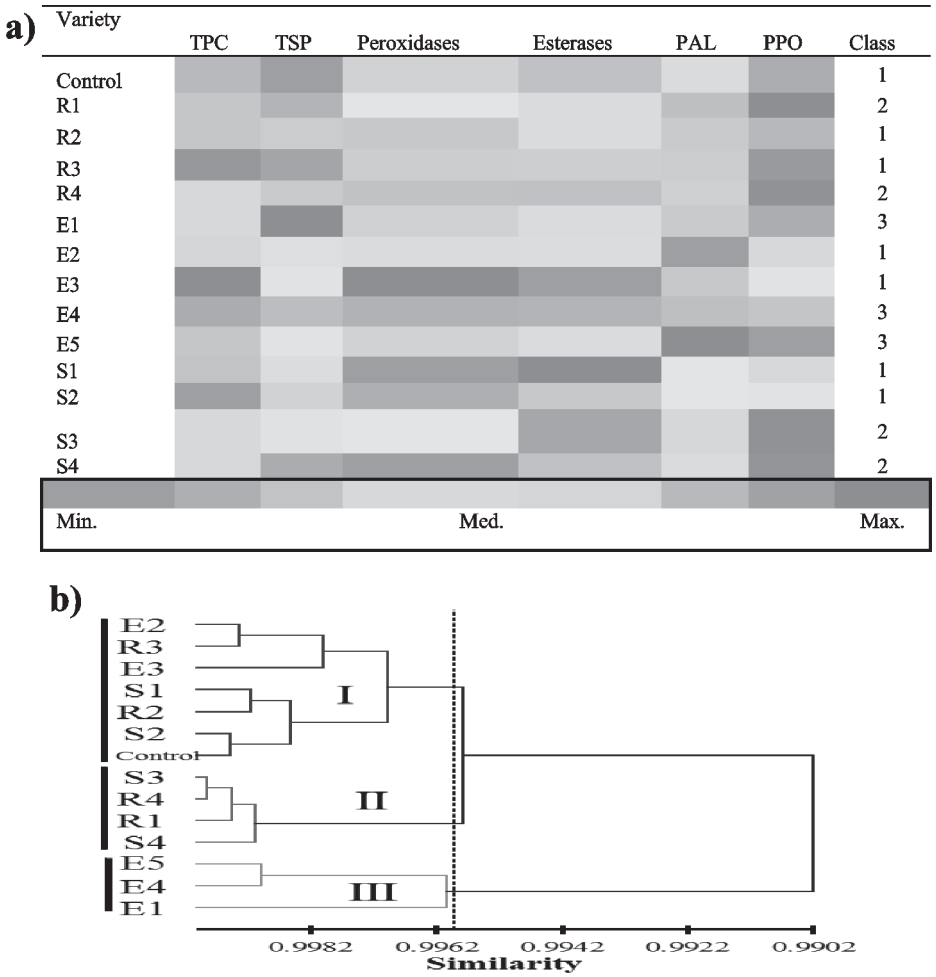

Fig. 3. Heat map of 14 selected plants showing maximum (Max.), median (Med.) and minimum (Min.) values of six parameters (legends are same as in Table 4.6) along with assigned Agglomerative Hierarchal Cluster (AHC) score at right which is based on un-weighted paired grouped average (UPGA); b) Similarity based AHC-clustering classifies 14 varieties in three clusters.

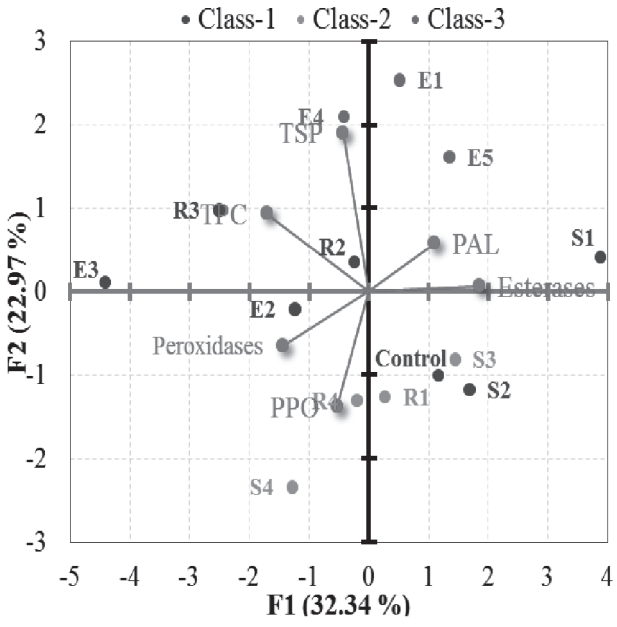

Fig. 4. Biplot of six parameters (legends are same as in Table 2) and 14 selected plants.

plants depicts that there exists a substantial homology in defence-response proteins of diverse plant species [57]. In an R/Avr combination direct physical interaction has also been reported [58]. This evidence suggest that R proteins along with other protein partners form complexes that participate in Avr perception and subsequent signal initiation [59]. In the present study, the increased total soluble protein contents in treated plants might be responsible for resistance against *A. solani* (Table 7). Batra et al. [60] reported the higher contents of total soluble proteins in the flag leaves and grains of aphid resistant genotypes of barley.

Phenolic compounds are highly reactive secondary metabolites that exhibit antifungal and antimicrobial activities. These are inbuilt natural chemical barriers that protect plants against biotic and biotic stresses [61], however, their magnitude of effectiveness vary making it difficult to predict their time of activeness. As oxidation of phenolic compounds makes them active; thus variation in oxidative conditions leads to variation in their activity [62]. In plants, the phenolic compounds are known as pre-formed antibiotics having an important

role of resistance against pathogens. They are called "phytoanticipin" and are stored as inactive forms inside the cell but pathogen attacks readily convert them to active antibiotics by hydrolysing enzymes (glycosidases) [63]. Phenolic compounds have favorable impacts due to their antioxidant activity. Certain phenolic compounds have combined effects rather than individual ones that enable them to reach concentration toxic to pathogens. Phenolic compounds (eg. Caffeic acid, Chlorogenic acid etc.) being reactive against pathogens are accumulated at faster rates in resistant plants as compared to susceptible [64]. Usually they are esterified with sugar moieties but most of them are bound to the cell wall or are stored in the vacuole, however, free phenolics appeared to be toxic as compared to bound one. These compounds are considered as precursors for lignin and suberin (can be polymerized into the cell wall) in the cell wall causing its thickening that not only plays a role for resistance against pathogens, but protection against other stress like drought and freezing. Phenolic compounds are considered as Pre-formed antibiotics in plants which play an important role for resistance against filamentous fungi. They are named as "phytoanticipin" to distinguish them from phytoalexins. These phytoanticipin are usually inactive in cell but as pathogens attack they become active as antibiotics by hydrolysing enzymes [65].

Under stress conditions, phenylalanine ammonia lyase (units/mg of protein) is induced that catalyses the first step of phenylpropanoid pathway (common step in yielding secondary metabolites). Similarly Flavonoids are synthesized via phenylpropanoid pathway [66]. Range of this diversity and quantity of phenylpropanoid products highlights the significance of PAL activity in plants. Biosynthesis of lignin is increased due to increase in the activity of PAL which are (lignin or lignin-like phenolic polymers) deposited rapidly in cell walls or cell wall perturbations [67]. Up-regulation of PAL genes activity in resistant cultivars of cotton, potato resistant against early blight and aphid resistant genotypes in Barley has well been reported [68]. Treatments that reduce PAL activity also reduce resistance against pathogens. According to Stadnik and Buchenauer [69], inhibition of PAL results in reduction of accumulation of autofluorogenic compounds that leads to suppress resistance induced by benzothiadiazole.

Polyphenol oxidase (PPO) oxidases phenolic compounds and are commonly induced during stress, therefore can interfere with plant physiological processes. In plants, level of PPO at early stages of growth give a clue for synthesis of phenolic compounds which can be correlated with resistance to stress [70]. Phenolic compounds are oxidized into quinones by PPO which is more toxic against pathogens. Therefore,

Table 7. Analysis of variance for Biochemical traits with completely randomized design (CRD).

Parameters	SOV	DF	MS	F
TPC	Variety	13	0.00729	5.24***
	Error	56	0.00139	
	Grand Mean	0.624	CV%	5.97
TSP	Variety	13	0.06057	53***
	Error	56	0.00114	
	Grand Mean	1.68	CV%	2.01
Peroxidases	Variety	13	0.01459	5.38***
	Error	56	0.00271	
	Grand Mean	1.085	CV%	4.79
Esterases	Variety	13	0.00411	6.22***
	Error	56	0.00066	
	Grand Mean	0.4307	CV%	5.97
PAL	Variety	13	0.0219	6.27***
	Error	56	0.00349	
	Grand Mean	1.14	CV%	5.17
PPO	Variety	13	0.66415	128***
	Error	56	0.00517	
	Grand Mean	3.7	CV%	1.94

*** : p-value <0.0001; TPC: Total Phenolic Compounds, TSP: Total Soluble Proteins

it becomes obvious that increase in the specific activity of PPO increases the production of toxic compounds of oxidation that induces resistance against pathogens [71]. A role of PPO in defense against pathogens may be due to the noticeable appearance of their reaction products in response to pathogen attack or any stresses [72]. Induction of PPO as a result of pathogen infection in young leaves has also been reported [73]. In leaves of Potato, cDNA for many polyphenol oxidases has been isolated and characterized. These genes are reported to be highly conserved with spatial differential expression. They are induced differentially against various stresses. Genotypes having over-expression of PPO genes show better resistance against diseases and are environmentally sound, potentially sustainable and cost effective. In selected genotypes of present investigation PPO and PAL exhibited similar patterns of increase.

Peroxidases are oxidative plant enzymes which can accept a broad spectrum of reducing substrates. They use hydrogen peroxide to catalyze oxidation of organic compounds. They have widely been reported to have a putative role in polymerization of lignin which not only have a role for defense against disease but also supportive functions in vascular tissues. Peroxidases not only remove toxic hydrogen peroxide from tissues but also participate in phenolic compounds biosynthesis and make intermolecular bonds in cell wall organization at pathogen infection sites. In plants, increase in specific activity of peroxidases during pathogen attack may be due to their involvement in biosynthesis and lignification of phenolic compounds and their accumulation in the cell wall making it an effective barrier against pathogens [74]. In the presence of H_2O_2, peroxidase carries out oxidation of phenolic compounds, the products of this oxidation accumulate in infected tissues of plants and provide resistance against pathogens. Therefore, peroxidases are believed to form potent antimicrobial systems in the presence of hydrogen peroxide. An increase in specific activity of peroxidases was recorded in all selected resistant genotypes in present study. Such increase in specific activity of peroxidases has repeatedly been reported. Shahbazi et al. [75] screened different varieties of potato against early blight and reported high peroxidase contents in resistant varieties. Patel et al. [76] reported an increase in specific activity of peroxidases during expression of early blight resistance in tomatoes. An enhancement in specific activity of peroxidases was reported by Solino et al., [77] during defense induction against culture filtrates of saprophytic fungi against early blight in tomato.

Esterases are fat splitting enzymes that hydrolyze esters into acid and alcohol. Esterases differ in host specificity and biological function. MeSA esterases hydrolyzes inactive methyl salicylate into salicylic acid which have a vital role in signal transduction pathway and help to activate systemic acquired resistance in plants [78]. According to Navarre and Mayo [79], potato plants are capable of tightly regulating free levels of salicylic acid that make tissues responsive against pathogen attack which is a prerequisite of disease resistance in plants. In the present investigation an enhancement in the activity of esterases was recorded in the majority of selected resistant lines as compared to control (Table 7). In 2001, Kim et al. [80] while working on capsicum reported an increase in activity of esterase which protected unripen fruit against anthracnose. Previous findings have also revealed high activity of esterses in smut resistant sugarcane plants. Volpi et al. [81], reported an increase in gene expression for pectin methyl esterase inhibitors which increases esterification of pectin methyl in cell walls to control fungal diseases in Wheat. All these reports clearly depict the role of esterases in increasing disease resistance response in plants.

RAPD markers are useful tools not only for identification/characterization of potato cultivars but also to estimate genetic relationships between them. Recently a genetic diversity among isolates of *Fusarium* [82] and *Rhizopus microspores* have been revealed using RAPD markers [83]. Different workers have reported the use of different numbers of RAPD-PCR based primers to detect polymorphism. Specific primers exhibit different banding patterns, some primers amplify to produce more bands and therefore are adequate to identify genotypes by RAPD technique. Some researchers used 24 primers in the past and reported 22 of them as polymorphic giving a total of 213 easily scorable bands out of which 65% bands were polymorphic. In the present study, seven out of ten RAPD markers showed polymorphism. Results obtained with this tool can lead toward new cultivar development which is resistant to pathogens. Under this assumption, plants with genetic variation were further tested for early blight resistance. In our study, plants were exposed to three types of mutations and their extracted DNA was analyzed using RAPD markers. Fourteen samples were classified into three clusters. Agglomerative hierarchical clustering (AHC) revealed that E-mutations were in a single cluster and the control sample was grouped with R4 in another cluster. The mutation S4, E5 showed maximum genetic distance and R4 was in the closest distance to control. RAPD marker M2, and M4 produced six and seven polymorphic bands, respectively and considered the best separation of all the induced variants. Such RAPD marker systems are efficiently applied for the diagnostics of potato blight and RAPD based markers were developed by [84]. In the present study, polymorphism information contents (PIC) ranged between 0.12 to 0.37 and percentage of polymorphism from 40 to 100%. This high polymorphism made it possible to obtain a better genetic diversity picture of 14 mutation-induced samples included in this study (Figure AHC-Clustering). To understand the genetic diversity, RAPD analysis is recently used in potato germplasm. Stress induced changes in potato has also been examined recently with the use of RAPD technique in potato [85]. To assess molecular and genetic diversity

in some other plant species such as onion and ginger, the RAPD markers system has successfully been applied [86]. Agglomerative Hierarchical Clustering (AHC) analysis has clearly revealed the genetic distinction among induced mutants and RAPD methodology efficiently detected such variations. Therefore, such products can further be utilized for bulk segregation analysis and Marker assisted selections.

Conclusion

Among seventeen media compositions examined, MS medium containing 3.0 mg/L of 2,4-D + 0.25 mg/L of BAP demonstrated maximum callus induction and proliferation phenomenon. After two year of field trials, four out of 63 in vitro screened lines of gamma irradiation; four out of 52 in vitro screened lines of sodium azide treated cultures and five out of 59 screened lines of EMS treated cultures were selected according to disease rating scales. During the current study, the high polymorphism made it possible to obtain a better genetic diversity picture of 14 mutation-induced samples.

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Conflict of Interest

The authors declare no conflict of interest.

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