Efficacy of cell line selection approach against *Phytophthora nicotianae* var *parasitica* (Dastur) Waterhouse causing buckeye rot of tomato

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ABSTRACT

In the present study, attempts were made to develop tolerant lines of tomato cv Solan Lalima against the pathogen *Phytophthora nicotianae* var *parasitica* causing buckeye rot, using in vitro selection approach. The highest callus survival and maximum shoot regeneration were attained on MS medium fortified with 1.0 mg/l 2,4-D and 0.5 mg/l Kn for cotyledon explants and 0.5 mg/l NAA with 1.0 mg/l BAP for leaf explants respectively. The proliferated callus was further exposed to selection pressure containing MS medium enriched with fungal culture filtrate (25%) that revealed 18.32 per cent mean callus survival and 34.59 per cent average shoot induction. The screened putative tomato tolerant somaclones against the targeted pathogen might be further subjected to molecular and agronomic evaluations including multi-location trials. Therefore, the strategy can be utilized as a promising selection method to generate disease-resistant plants of this commercially important crop.

Keywords: Tomato; cell line selection; fungal culture filtrate; *Phytophthora nicotianae*; buckeye rot somaclonal variation

INTRODUCTION

Tomato (*Solanum lycopersicum* L) is the largest vegetable crop in the world next to potato with its significant importance in processed products (Sharma et al 2015). Its production is hampered by a number of fungal infections (late blight, early blight, septoria leaf spot, fusarium wilt, powdery mildew, anthracnose, verticillium wilt, buckeye rot etc). Among these, buckeye fruit rot caused by the fungus-like *Phytophthora nicotianae* var *parasitica* (Dastur) Waterhouse becomes an endemic disease during high humidity ($\geq$60%) and warm temperature (20-25ºC) in the summer and rainy seasons. Fruit with a buck's eye pattern of dark brown rotting and alternate concentric rings of light and dark brown discolouration are typical symptoms of the ailment (Gupta et al 2022).

The primary sporangium (lemon-shaped tip) emerges from the stomata and further stages result in the formation of motile zoospores. The sporangia are dispersed through irrigation water, splashing rains from soil to fruit, runoff water, farm equipment and by the workers.

Various available management methods have been used due to the disease's rapid inoculum buildup and extensive nature, such as proper soil drainage, mulching of soil beds, multiple cropping programmes, treatment of suitable antifungal agents (mancozeb, metalaxyl and chlorothalonil) and fumigation. Moreover, the chemical treatment also affects produce quality and results in associated health risks if used in random way (Kumari et al 2022).

With some degree of success, improved management and traditional breeding have been aimed at controlling this disease of tomato. Similarly, through biotechnological approaches, the most widely used strategy to engineer fungal disease resistance has been over-expression of chitinases and glucanases but they have their own regulatory issues (Sharma et al 2012).

Selecting somaclonal variants, produced via in vitro tissue culture techniques, can be used as a substitute for traditional breeding to gain useful genetic variation in horticultural crops (Goel at al 2007, Bhardwaj et al 2012, Shaunak et al 2023). Using somaclonal variations, plants are also anticipated to possess several desired traits such as larger fruit size, more fascinating blossom texture, improved taste and improved productivity and it has become possible to successfully achieve resistance to biotic or abiotic stresses without altering other desired characters (Jain 2001). The advancement of in vitro selection technology will open up new possibilities for increasing the stress tolerance of plants vital to maintain environmental sustainability and food supply. Therefore, the present study was focused on the development of tolerant lines of tomato cv Solan Lalima against the pathogen *P nicotianae* var *parasitica* causing buckeye rot using in vitro selection approach.

MATERIAL and METHODS

Source of plant material: The tomato cv Solan Lalima seeds were procured from the Department of Seed Science and Technology, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh in order to harvest explants. Explant is a limiting factor in plant regeneration (Sharma et al 2011), therefore, fully expanded, healthy and young cotyledons and leaves were excised from apical portions and placed with their abaxial sides oriented downward on MS (Murashige and Skoog 1962) medium with different concentrations and combinations of growth regulators for callus induction as suggested by Bharti et al (2018).

Isolation and identification of pathogen (*P nicotianae* var *parasitica*): The fungal culture of pathogen *P nicotianae* var *parasitica* was isolated from diseased fruit of tomato obtained from the field of Department of Plant Pathology, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh showing the buckeye rot symptoms (Fig 1). For cultural and morphological identification, the cultures were examined under a microscope and the pathogen was identified by comparing characteristics such as colony colour, colony appearance, hyphal size and colour and sporangia size and shape with the accepted standards outlined by Waterhouse (1963) and finally confirmed using molecular analysis.

Preparation of culture filtrate and selective medium: In order to prepare culture filtrate (CF), the pathogen was replicated on corn meal broth medium by adding 1-2 bits (5 mm^2) of actively developing mycelium. Inoculated flasks were kept stationary in an incubator at 25ºC until full mycelium development. By combining the CF (25%, v/v) with a sterile molten MS regeneration medium, the selective medium was prepared (Bharti 2018). The purified culture filtrate of the pathogen (filtered through 0.22 m) was combined with sterilized molten MS medium and the suitable growth regulators to create the selection medium, which had v/v concentrations of 0, 20, 22.5, 25, 27.5, 30 and 32.5 per cent.

Fig 1. Tomato fruits showing buckeye rot disease symptoms

Selection of tolerant calli and in vitro regeneration: The tolerant calli were selected according to the calli survival rate after both continuous and discontinuous selection cycles. In continuous selection cycle, calli were proliferated on a selective medium providing a sub-culture every 30 days for two to three months, whereas, under discontinuous selection cycle, calli were cultured on selective medium for 30 days and then sub-cultured on control medium for further growth. Shoot regeneration from the tolerant calli was attempted on shoot regeneration MS medium supplemented with 1.00 mg/l BAP and 0.50 mg/l NAA (Bharti 2018). At various stages of sub-culturing, shoots between 1.5 and 2.0 cm in length were excised and transferred to MS media enriched with 0.50 mg/l IBA.

Statistical analysis: Completely randomized design (CRD) was applied to the data for the experimental parameters. The frequency of regenerant survival on selective medium, the impact of selection cycles and the culture filtrate on callus survival were studied using two-way factorial analysis.

RESULTS

Well-developed green and friable calli were obtained using leaf explants on MS regeneration medium containing 1.00 mg/l BAP and 0.50 mg/l NAA within 45 days of culturing. Using cotyledon as an explant, several combinations of auxins (IBA, NAA and 2,4-D) and cytokinins (BAP and TDZ) were also attempted, considering the late and poor percentage of callus production and the frequency of shoot regeneration (Bharti 2018). The MS medium with 0.5 mg/l Kn and 1.0 mg/l 2,4-D was proven to have the best callus induction rate with maximum 73.41 per cent callus induction from cotyledon explant out of all the tested growth regulator combinations (Table 1). After 45 days of culture, the developed calli were observed to be green and friable.

Isolation and identification of pathogen: White to creamy mycelial growth with slight yellow pigmentation in a rosette pattern was obtained after 6 days of inoculation. Aseptate hyphae and lemon shaped sporangial growth were observed under microscope (Fig 2). According to the phylogenetic analysis obtained after molecular analysis, the microbe (*Phytophthora*) was found to be closely similar to KJ494909.1 *P nicotianae* isolate SX-1 and other isolates of *Phytophthora* sp.

Selection of tolerant calli: The developed calli were further subjected to a selective medium containing MS medium augmented with various percentages of culture filtrate, ranging from 20 to 32.5 per cent (Table 2) following continuous and discontinuous selection cycles for the selection of tolerant callus. To achieve the optimum selection pressure that permitted the recovery of tolerant lines, the selection was done at a concentration of 25 per cent culture filtrate where average callus survival of 18.32 per cent was obtained. All the calli in the continuous selection approach turned brown and eventually died, whereas, the calli in the discontinuous method appeared to turn brown. It was found that a small number of cells restored their potential to survive and propagate into tolerant plants under several cycles of discontinuous approach. Due to the harmful metabolites present in the culture filtrate, it was determined that the survival rate of calli dropped as the proportion of culture filtrate increased. It was also observed that some cells gained the ability to develop and survive under stress conditions and led to the development of tolerant plants.

Shoot regeneration from selected calli: The morphogenetic response of the calli (weight, type, colour and size) on selected and control media was recorded after the selection of tolerant calli. Selected calli were cut into small pieces and grown on MS medium supplemented with 1.00 mg/l BAP and 0.50 mg/l NAA to produce shoots from tolerant calli (Bharti 2018). Average weight of the tolerant callus was 0.50 g after selection, which increased during further sub-culturing ie to 1.37 g on control standardized media up to four sub-cultures. After 90 days of sub-culture, the maximum average shoot induction was seen (34.59%) with the maximum average number of shoots per callus clump (2.58) and the maximum average shoot length (1.56 cm), while there was no induction of shoots seen in the early stages of sub-culturing (Table 3) (Fig 3). At various stages of sub-culturing, in vitro propagated shoots with length ranging from 1.5 to 2.0 cm were excised and transplanted to MS media supplemented with 0.50 mg/l IBA for root induction that appeared after 4 weeks of culturing (Fig 3).

DISCUSSION

The results on callus regeneration from the leaf and cotyledon of tomato are consistent with the findings of Bharti (2018) and confirm the reproducibility of the results. Similar research has been done on the induction of callus from leaf and cotyledon explants in

Table 1. Efficacy of various cytokinins and auxins on callusing and shoot regeneration using cotyledon as explants

Cytokinins (mg/l)			Auxins (mg/l)			Callus induction (%)	Morphogenetic response of callus of cotyledon explant			Average shoot regeneration (%)	Average number of shoots/explant	Average shoot length (cm)
BAP	Kn	TDZ	NAA	IBA	2,4-D		Type	Colour	Growth			
–	–	–	–	–	–	0.00	–	–	–	0.00	0.00	0.00
0.5	–	–	–	0.5	–	30.40 ± 0.52	C	PY	+	0.00	0.00	0.00
0.5	–	–	–	1.0	–	48.55 ± 0.75	C	PY	+	0.00	0.00	0.00
0.5	–	–	–	1.5	–	56.36 ± 0.73	F	PY	+	0.00	0.00	0.00
0.5	–	–	–	2.0	–	55.42 ± 0.65	F	LG	++	38.30 ± 0.64	3.34 ± 0.33	1.27 ± 0.33
1.0	–	–	–	0.5	–	63.38 ± 0.59	F	LG	++	41.24 ± 0.64	2.67 ± 0.33	1.94 ± 0.08
1.0	–	–	–	1.0	–	49.37 ± 0.63	C	PY	+	29.09 ± 0.51	2.34 ± 0.33	1.67 ± 0.12
1.0	–	–	–	1.5	–	46.67 ± 0.48	C	PY	+	0.00	0.00	0.00
1.0	–	–	–	2.0	–	39.59 ± 0.46	F	B	+	0.00	0.00	0.00
–	0.5	–	–	–	0.5	69.70 ± 0.57	F	G	+++	55.54 ± 0.41	2.34 ± 0.33	1.30 ± 0.15
–	0.5	–	–	–	1.0	73.41 ± 0.80	F	G	+++	71.81 ± 0.46	4.67 ± 0.33	1.87 ± 0.23
–	0.5	–	–	–	1.5	67.54 ± 0.59	F	G	++	62.57 ± 0.75	3.37 ± 0.33	2.23 ± 0.13
–	0.5	–	–	–	2.0	58.50 ± 0.54	F	LG	++	55.65 ± 0.41	2.37 ± 0.33	1.60 ± 0.11
–	1.0	–	–	–	0.5	50.55 ± 0.70	C	LG	++	47.71 ± 0.65	1.67 ± 0.33	0.84 ± 0.16
–	1.0	–	–	–	1.0	46.37 ± 0.67	C	LG	++	0.00	0.00	0.00
–	1.0	–	–	–	1.5	42.52 ± 0.56	C	PY	+	0.00	0.00	0.00
–	1.0	–	–	–	2.0	34.58 ± 0.49	C	PY	+	0.00	0.00	0.00
–	–	0.25	0.5	–	–	53.74 ± 0.58	C	LG	+	0.00	0.00	0.00
–	–	0.25	1.0	–	–	55.43 ± 0.58	F	LG	+	0.00	0.00	0.00
–	–	0.25	1.5	–	–	60.36 ± 0.42	F	LG	+	0.00	0.00	0.00
–	–	0.25	2.0	–	–	67.30 ± 0.59	F	LG	++	0.00	0.00	0.00
–	–	0.5	0.5	–	–	46.60 ± 0.50	C	LG	+	0.00	0.00	0.00
–	–	0.5	1.0	–	–	49.43 ± 0.70	C	LG	+	0.00	0.00	0.00
–	–	0.5	1.5	–	–	39.29 ± 0.57	C	LG	+	0.00	0.00	0.00
–	–	0.5	2.0	–	–	34.24 ± 0.60	C	LG	+	0.00	0.00	0.00
0.5	–	–	–	–	0.5	48.36 ± 0.58	C	LG	+	0.00	0.00	0.00
0.5	–	–	–	–	1.0	60.48 ± 0.64	F	LG	++	0.00	0.00	0.00
0.5	–	–	–	–	1.5	62.56 ± 0.52	F	LG	++	29.49 ± 0.63	0.67 ± 0.33	0.94 ± 0.07
0.5	–	–	–	–	2.0	63.32 ± 0.64	F	LG	++	32.68 ± 0.64	1.67 ± 0.33	1.44 ± 0.08
1.5	–	–	–	–	0.5	49.26 ± 0.55	C	PY	+	26.41 ± 0.46	1.34 ± 0.3	1.25 ± 0.22
1.5	–	–	–	–	1.0	46.37 ± 0.38	C	PY	+	0.00	0.00	0.00
1.5	–	–	–	–	1.5	39.75 ± 0.54	C	B	+	0.00	0.00	0.00
1.5	–	–	–	–	2.0	36.61 ± 0.56	C	B	+	0.00	0.00	0.00
						1.65				0.85	0.61	0.24

Data taken after 45 days; BAP: 6-Benzyl amino purine, Kn: Kinetin, TDZ: Thidiazuron, NAA: Naphthalene acetic acid, IBA: Indole-3-butyric acid, 2,4-D: 2,4 Dichlorophenoxyacetic acid, CIM: Callus induction medium, C: Compact, F: Fragile, G: Green, LG: Light green, PY: Pale yellow, B: Brown, +: Slow, ++: Moderate, +++: Fast, –: No response

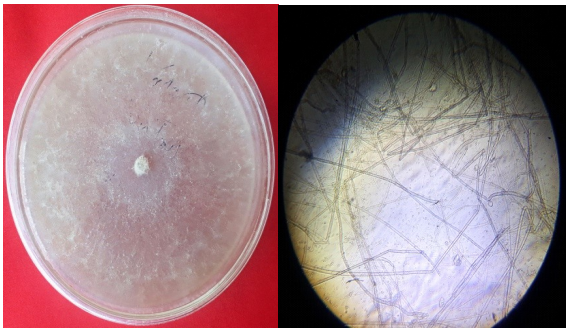

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Fig 2. Isolation and identification of *Phytophthora nicotianae* A) Pure culture of pathogen on corn meal agar, B) Microscopic view of aseptate hyphae

Table 2. Effect of different concentrations of culture filtrate (CF) of *P nicotianae* var *parasitica* on survival of tomato callus after different selection cycles (discontinuous selection)

Treatment	CF concentration (%)	Average survival (%)	
		After 1st selection cycle	After 2nd selection cycle
Control	0	100.00 ± 0.00	100.00 ± 0.00
T_1	20.0	41.34 ± 0.70	39.48 ± 0.70
T_2	22.5	32.44 ± 0.43	30.42 ± 0.60
T_3	25.0	23.70 ± 0.63	18.32 ± 0.58
T_4	27.5	9.36 ± 0.61	7.35 ± 0.50
T_5	30.0	4.37 ± 0.21	2.07 ± 0.51
T_6	32.5	0.00	0.00
$CD_{0.05}$		1.55	1.46

Data taken after 30 days; CF: Culture filtrate

various tomato varieties, including work by Devi et al (2008) who supplemented MS medium with 3.0 mg/l BAP and 2.5 mg/l IAA and found that further increase in growth regulator concentrations resulted in a decrease in callusing, revealing a toxic hormonal effect on callus. Likewise, in order to generate longer and healthy shoots, growth regulators have been shown to be effective in in vitro tomato shoot regeneration. Deb et al (2019) used MS medium supplemented with 0.2 mg/l NAA and 3.0 mg/l BAP to achieve 73.33 per cent shoot regeneration. They concluded that cytokinins, in contrast to auxins, have a far greater impact on shoot regeneration and are primarily in charge of cell division and the differentiation of shoots from callus. In the present investigation, shoot regeneration from callus was achieved using MS media supplemented with 1.00 mg/l BAP and 0.50 mg/l NAA. It was shown that the proportion of growth regulators required for shoot induction varied with the tissue and was directly associated with the amount of hormones generated at endogenous levels inside the cells of the explant.

Hardham (2001) primarily used the monopodial branching habit of the sporangiophore and the papillate sporangia that release zoospores in free water to characterize *Phytophthora* sp. Similarly, Singh and Islam (2010) identified the pathogen during their investigation on black shank disease caused by *P nicotianae* by looking at the colour of the pathogen's colony and aseptate hyphae, which could be spotted using a microscope. Gupta et al (2022) described identification features of the pathogen as growth in the form of light and dark rings on green tomato fruits and climatic requirement ie humidity of about 60 per cent and temperature 20-25°C for the formation of sporangia and zoospores.

Sharma et al (2008) used the continuous and discontinuous methods of selection to carry out an in vitro regeneration experiment on asiatic lily that was resistant to *Phytophthora cactorum* culture filtrate. By using a discontinuous approach of selection, tolerant callus was produced at a concentration of 20 per cent

Table 3. Effect of sub-culturing on tolerant callus proliferation and shoot regeneration at four weeks interval

Sub -culture	Medium	Callus weight (g)	Callus type	Callus colour	Callus growth	Average shoot induction (%)	Average number of shoots/callus clump	Average shoot length (cm)
I	MS + 1.00 mg/l BAP + 0.50 mg/l NAA + 25% CF	0.50 ± 0.03	C	LG	+	0.00	0.00	0.00
II	MS + 1.00 mg/l BAP + 0.50 mg/l NAA	0.62 ± 0.05	C	LG	+	14.47 ± 0.57	0.31 ± 0.54	0.34 ± 0.21
III	MS + 1.00 mg/l BAP + 0.50 mg/l NAA	1.03 ± 0.06	C	LG	++	26.70 ± 0.70	1.26 ± 0.53	0.86 ± 0.29
IV	MS + 1.00 mg/l BAP + 0.50 mg/l NAA	1.37 ± 0.08	F	LG	+++	34.59 ± 0.71	2.58 ± 0.55	1.56 ± 0.41
V	MS + 1.00 mg/l BAP + 0.50 mg/l NAA	0.98 ± 0.07	F	LG	++	19.39 ± 0.59	1.68 ± 0.38	0.85 ± 0.29
$CD_{0.05}$		0.20				1.79	1.56	0.94

C: Compact, F: Fragile, LG: Light green, +: Slow, ++: Moderate, +++: Fast

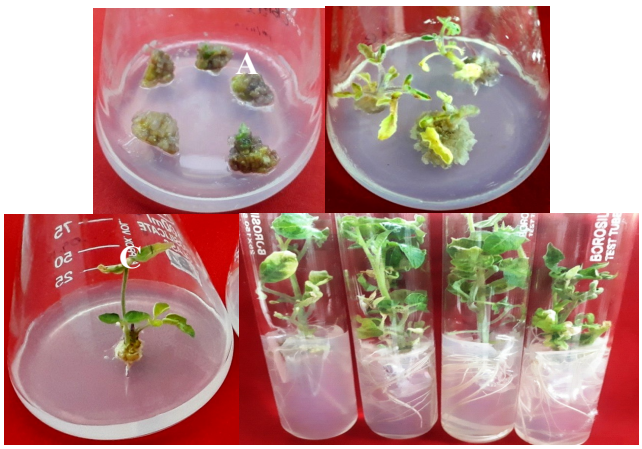

Fig 3. In vitro shoot regeneration and root induction from tolerant calli

fungal culture filtrate. Moreover, Tripathi et al (2008) confirmed the continuous and discontinuous methods of in vitro selection of tolerant lines on *Allium cepa* to combat the purple blotch brought on by *Alternaria porri*. It was determined that by using the discontinuous approach of selection, a greater number of tolerant plants could be acquired as compared to the continuous selection.

It can be concluded that an effective method for developing the tolerant somaclones against *P nicotianae* var *parasitica* (Dastur) Waterhouse, that causes buckeye rot in tomato using culture filtrate, has been demonstrated. This method has been proved successful in exhibiting partial resistance according to preliminary studies. The putative tomato tolerant somaclonal variants tolerating selection pressure may be further subjected to molecular and agronomic evaluations including multi-location trials. Thus the strategy can be utilized as a promising selection method to generate disease resistant plants of this commercially important crop.

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