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In vitro cell selection and its molecular confirmation in tomato against buckeye rot (*Phytophthora nicotianae* var. *parasitica*)

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Abstract
Among various fungal diseases buckeye rot is a major constraint in tomato cultivation and the disease is present in almost all the tomato growing areas of the world. The aim of present study was to evaluate the effectiveness of *in vitro* test in which culture filtrate of *Phytophthora nicotianae* var. *parasitica* was used to select the tolerant calli against buckeye rot disease. *Murashige and Skoog* medium supplemented with 3.0 mg/l Naphthalene acetic acid (NAA) + 1.0 mg/l 6-Benzylaminopurine (BAP) was used for callus induction and multiplication. The calli derived from the cotyledon explant of *in vitro* raised aseptically grown seedling were selected against the different concentrations (1-25%) of culture filtrate of *Phytophthora nicotianae* var. *parasitica.* Calli survival at 20% as selective dose of culture filtrate was 30.64% and survived calli were subcultured on multiplication medium (NAA 3mg/l + BAP 1mg/l) without culture filtrate. After multiplication, the calli were again culture on 20% selective medium and this process was repeated two to three times and tolerant calli were selected. The selected calli were transferred to the previously standardized regeneration medium (MS medium supplemented with 2.75, 3.0 mg/l BAP). Initially the callus turned to slightly raised green nodulation on this growth regulator which later leads to shoots formation. Molecular studies revealed that the selected tolerant calli were different at genomic level from the normal callus and it ascertains that indeed the resistance adaptation observed is due to genetic changes. Finally, it was concluded that resistant plantlets regenerated from tomato cv. Solan Vajr callus can be used for cell selection and will benefit the farmers to encounter with huge crop losses due to buckeye rot.

Keywords: Tomato; selection; culture; calli

Introduction

Tomato (*Solanum lycopersicum* L.), belonging to family Solanaceae, is a widely grown vegetable all over the world (Agrios 2005) [2]. It is rich in vitamins (vitamin A and C), minerals and lycopene (Zhang *et al.* 2009) [30]. In Himachal Pradesh, the Solan Vajr is a high yielding and drought tolerant promising commercial cultivar but this cultivar is susceptible to buckeye rot disease. Among various fungal diseases buckeye rot is a major constrain in tomato cultivation and the disease is present in almost all the tomato growing areas of the world. Buckeye rot of tomato is caused by *Phytophthora nicotianae* var. *parasitica.* This disease causes 35-40 per cent losses, which may rise with the severity of the disease depending upon the favourable weather conditions i.e. prolonged warm and wet weather, and poor soil drainage (Gupta *et al.* 2005) [10]. Symptoms of this disease mainly appear on fruits touching the soil surface. Brownish spot appears on the fruit at the point of contact with soil and white flocculent superficial growth of the fungus also develops profusely on the diseased fruits. Conventional breeding programs including extensive intermating and screening campaigns help breeders to improve cultivars, however, this is limited by inherent difficulties, open pollination, high level of heterozygosity and poor fertility of F1 hybrids. The only measures used to control this disease are by ensuring proper soil drainage, avoiding soil compaction, use soil fumigation to disinfect heavily infested soils, rotation on a three to four year basis with crops other than those belonging to the tomato family, reducing the contact between fruit and soil by using stakes or mulches and applying fungicides may also control the disease. But there is no resistant cultivar available for this disease. As an alternative to conventional breeding, Plant tissue culture is an important tool in biotechnology for selecting resistant cultivar for a particular variety. In tissue culture one approach for obtaining useful genetic variation is to

select for somaclonal variants (Larkin and Scowcroft, 1981) [14] generated by tissue culture techniques. In order to screen target characters it is essential to have efficient selection agents (Liu *et al.* 2005) [15]. The use of pathogen toxins as selective agents at the tissue culture step might be a source of variability that can lead to the selection of individuals with suitable levels of resistance to the toxin or to the pathogen amongst the genetic material available (El Hadrami *et al.* 2005) [5]. This approach presumes that tolerance operating at unorganised cellular level can be carried over to whole plant. Since no work has been reported so far for *in vitro* selection against *Phytophthora nicotianae* var. *parasitica*, the present investigation was undertaken for *in vitro* selection of tomato cv. Solan Vajr callus against culture filtrates of *Phytophthora nicotianae* var. *parasitica* and attempts to regenerate selected callus was done, along with molecular marker assisted confirmation of polymorphism.

2. Material and Methods

2.1 Plant material

High yielding commercial tomato cv. Solan Vajr, susceptible to buckeye rot was used to carry out present study. Seed of the plant were obtained from the Department of Vegetable Science, University of Horticulture and Forestry, Solan, India.

2.2 Establishment of fungal culture and pathogenicity test

Phytophthora nicotianae var. *parasitica* was isolated from the infected fruit of tomato on Corn Meal Agar (CMA) medium. The culture was incubated at 25°C for 7-10 days till uniform fluffy growth was obtained. Thereafter the culture plates with pathogen were covered properly and kept at low temperature (4°C) to stop further growth. The purified culture of the *Phytophthora nicotianae* var. *parasitica* was maintained at our laboratory for further use.

The identification of the fungus was done initially by observing the morphological characteristic of fungus on the culture plate (Fig. 1) and then the fungal culture was observed under the light microscope. The characteristic highly branched and multinucleated mycelium are clearly visible in the magnified microscopic view of fungal culture of *Phytophthora nicotianae* var. *parasitica* under the microscope (Fig.2).

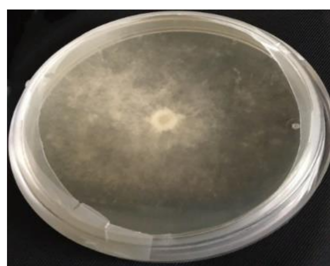

Fig 1: Isolated culture of *Phytophthora nicotianae* var. *parasitica* on CMA medium after one week of culturing

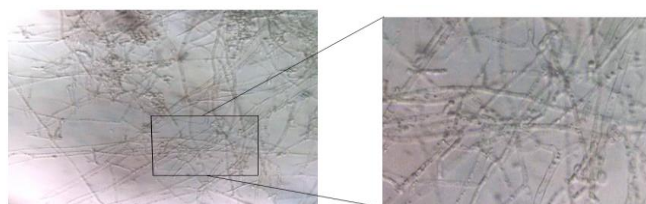

Fig 2: Fungal culture under the microscope showing the characteristic highly branched and multinucleated mycelium of isolated *Phytophthora nicotianae* var. *parasitica*

To test the pathogenicity of fungus, tomato fruits were inoculated with fungal suspension of *Phytophthora nicotianae* var. *parasitica*. Autoclaved distilled water and nutrient broth were taken as control treatments. The fruits were covered with perforated polythene bags for maintaining humidity. After three days of inoculation, the symptoms of the disease were observed on the inoculated fruits in the form of brown coloured concentric rings, whereas no such symptoms were observed in non-inoculated fruits in case of control treatments.

2.3 Prepration of culture filtrate of pathogen

The culture filtrate was prepared by inoculating pathogen in 250 ml flasks containing 150 ml of corn meal broth and incubated in incubator shaker with constant shaking (100 rpm) at 27±2°C for 25 days. After the 25-30 days the fungus cultures were used for the preparation of culture filtrate using three distinct steps: 1) At the end of incubation period, suspension was *filtered* through ordinary filter paper, 2) Then the filtrate was centrifuged at 10,000 rpm for 15 minutes, 3) Final filtration through nylon membrane filter (0.22 µm) was done. After filter sterilization, the culture was kept in the culture room for 48 hours to allow the growth of fungus and to check if there were any fungal spores in the culture filtrate. Thereafter the culture filtrate was used for the preparation of selective medium only if it did not contain any fungal mycelia. The pure culture filtrate was transparent in appearance and was stored at 4 °C for further use.

2.4 Medium for screening of tolerant cells

The medium used for selection was prepared by mixing the purified culture filtrate of the pathogen with sterilized molten Murashige and Skoog (MS) medium supplemented with 3.0 mg/l NAA and 1.0 mg/l BAP, medium used for callus induction and multiplication of tomato by Kumar *et al.* (2017), so as to obtain v/v concentration of 0, 1, 5, 10, 15, 20, 25 per cent, respectively. The medium was thoroughly mixed with culture filtrate and poured in pre-sterilized petriplates under aseptic conditions.

2.5 Cell plating on the selective medium

Callus was cut into small pieces of about 20 mg each and then inoculated into selective medium of different concentrations in laminar air flow chamber followed by incubation in the culture room at 25 ± 2°C under 16 hour photoperiod and eight hour dark. The growth of the cells was monitored by their ability to divide and form colonies.

2.6 Selection and isolation of tolerant callus against the pathogen

The highest concentration of culture filtrate was selected at which the calli survived. The green and light brown colour of the callus depicted that cells were alive whereas dark brown colour indicates that the cells were dead. The survived calli were subcultured on multiplication medium (NAA 3mg/l + BAP 1mg/l) without culture filtrate. After multiplication, the calli were again culture on the selected medium of concentration on which selection was made in the previous step and this process was repeated two to three times and tolerant calli were selected.

2.7 Attempts to regenerate shoots from the selected and normal callus

Regeneration of shoots was attempted from normal callus on MS medium supplemented with different growth regulators,

predominantly cytokinins. After that regeneration was attempted for selected callus.

2.8 Molecular studies

2.8.1 Genomic DNA isolation along with quality and quantity assessment

Genomic DNA from the selected and normal callus was isolated following CTAB method, followed by purification using chloroform – phenol treatment with suspension and precipitation using absolute alcohol. Finally the pellet was dissolved in Tris EDTA (TE) buffer (10mM tris HCl and 1M EDTA, pH 8) and stored at 4°C till further use.

The genomic DNA was electrophoresed on 0.8% agarose gel, stained with ethidium bromide (0.5 µg/ml), at 80 V – 100 V and 70 mA for two hours in 1X TAE buffer for quality assessment which was judged on the basis of whether sample DNA formed a single high molecular weight band or smear.

The quantity of DNA was assessed by UV/VIS spectrophotometer (Perkin Elmer, Hyderabad, India). DNA purity assessment was done by calculating absorbance ratio at 260 nm and 280 nm using the formula given below, which ranged between 1.4 and 1.8, indicating good quality of DNA.

$$\text{DNA } (\mu g/ml) = \frac{OD(260) \times \text{dilution factor} \times 50}{1000}$$

2.8.2 ISSR studies for selected and normal callus

DNA isolated from different sample of selected and normal callus was amplified by Polymerase Chain Reaction (PCR) using different ISSR primer. Optimum concentration of various components used were as: PCR buffer IX, $MgCl_2$ 2mM, dNTP mix 1mM, primer 10 picomoles, template DNA 30 ng, and Taq DNA polymerase 1U for 20 µl reaction mixture. The thermal profile used for amplification was programmed as follows: Initial denaturation at 94 oC for 2 minutes, followed by 40 cycles each of denaturation at 94 oC for 10 seconds, annealing according to primer Tm for 30 seconds, extension at 72 oC for 65 seconds and thereafter final extension at 72 oC for 10 minutes. The amplified DNA then was mixed thoroughly with 6X loading dye and then electrophoresed in 2% agarose gel in 1X TAE buffer. The gel was run at constant voltage at the rate of 5V/cm under submerged conditions for about three hours.

3. Statistical analysis

The recorded data were subjected to completely randomized design (CRD) to fulfill the statistical analysis purposes. Results were subjected to analysis of variance (ANOVA) using CRD. Molecular data was analysed by the polymorphism shown by primers.

4. Results and Discussion

4.1 *In vitro* selection of callus against culture filtrate of *Phytophthora nicotianae* var. *parasitica*

4.1.2 Pathogenicity test

The tomato fruits were inoculated with the culture of fungus with the help of the syringe. Symptoms of infection started appearing after 4-5 days. Initially brownish spots started appearing on the fruit. The spot further enlarged and developed into a lesion with a characteristic target-like pattern of concentric rings of narrow dark brown and wide light brown bands. These symptoms did not appear in the control plant.

4.1.3 *In vitro* cell selection

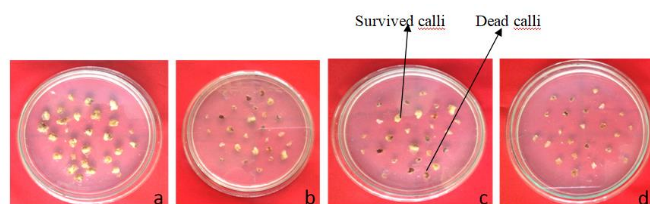

Fig 3: Selection of callus tolerant to culture filtrate of pathogen

The normal calli in the selection experiment were cultured on the medium containing different concentrations (v/v) of culture filtrate of pathogen. The survival of calli at each concentration was recorded. 97.58% survival of callus was observed at one per cent of culture filtrate and with the increase in the concentration of culture filtrate cell survival rate decreased i.e. 92.76%, 71.26%, 40.60%, 30.64% at 5%, 10%, 15%, 20% of culture filtrate, respectively (Table 1; Fig. 3). Callus found to be tolerant to 20% of culture filtrate, was further multiplied on callus initiation and maintenance 20% of culture filtrate was divided into five tolerant lines and these tolerant line were alternatively cultured on selective and normal medium (non-toxic medium). It is Fig.3. Selection of callus tolerant to culture filtrate of pathogen a) Callus on normal MS + 3.0 mg/l NAA + 1.0 mg/l BAP, b) Callus on selective medium with 15 percent of culture filtrate, c) Callus on selective medium with 20 per cent of culture filtrate, d) Callus on selective medium with 25 percent of culture filtrate reported in the present study that in the discontinuous approach for *in vitro* selection, the resistant lines retained their tolerance to toxin. It may be due to the calli regaining their viability.

Table 1: Effect of different concentrations of culture filtrate of *Phytophthora nicotianae* var. *parasitica* on per cent survival of callus of tomato

Concentration of culture filtrate	Survival of calli (%)
0	100.00(90.00)
1	97.58(81.27)
5	92.76(74.38)
10	71.26(57.56)
15	40.60(39.57)
20	30.64(33.59)
25	0.00(0.00)
C.D.	2.00
S.E.	0.65

Values in parentheses are arcsine transformed

The cell survival decreased to zero per cent when concentration of culture filtrate was increased to 25%. Therefore, cell selection was done at 20% culture filtrate concentration, where cell survival was 30.60%. At 20% culture filtrate, calli first turned brown in 8-10 days and after 15 days a few cells start growing out of these brown calli.

Several culture systems of differing levels of complexity may be used as the basis of *in vitro* selection. These are cell suspension cultures, calli, embryoids, microspores, *in vitro* regenerated plants etc. There are advantages as well as disadvantages associated with each type of tissue and no one system can be singled out as being ideal. The final choice therefore depends upon the type of variant to be selected, the selection strategy to be applied and tissue culture capability of the crop, so that high frequency of regeneration must be obtained from that tissue (El-Kazzaz and Ashour, 2004;

Tripathi *et al.* 2008; Esmaiel *et al.* 2012) [6, 27, 7]. Culture filtrates in several cases have shown the presence of extracellular non-specific phytotoxins that determine pathogenicity Nachimas *et al.* 1979 [17]; Pennisi *et al.* 1988 [20]; Sesto *et al.* 1990 [24]. Also, a correlation is must between the *in vitro* and *in vivo* responses of the host to the infection caused by a pathogen or the toxin to be utilized for the selection of disease-resistant genotypes. In our study browning of callus due to necrosis after application of a culture filtrate of pathogen confirmed that there is some factor present in the culture filtrate which is responsible for causing disease symptoms similar to those caused by the pathogen in natural infection conditions. These studies are supported by the fact that an important factor in disease development is the production of a toxin that is released when the fungus is grown on an appropriate liquid medium (Plich and Rudnicki, 1979) [21].

In vitro selection is practical when there is a correlation between the response of cell in culture and that of the whole plant. Heritable resistance to pathotoxins have been reported for *Phytophthora infestans* in potato (Behnke 1980) [3], where resistant plants were regenerated. Sacristan (1982) [22] regenerated plants with reduced susceptibility to *Phoma lingam* from callus and embryogenic culture of haploid rape. The regenerants survived exposure to the toxin produced by the fungus.

The possibility of *in vitro* selection of disease resistance was first tested by Carlson (1973) [4] for wild fire disease of tobacco caused by *Pseudomonas syringae* pv. *tabaci*. Later Genegenbach *et al.* (1997) [9] used t-toxin of *Helimentosporium maydis* to select T-toxin resistant cell lines of corn. Behnke (1980) [3] was the first to perform the cell selection by using crude culture filtrate of *Phytophthora infestans* on calli of diploid potato clones. As perusual of literature reveals that most of the work, in various plant spieces, on cell selection has been done in fungal pathogens such as *Fusarium oxysporum* (El-Kazzaz and Ashour 2004; Nasir *et al.* 2008; Hashem *et al.* 2009; *Esmaiel et al. 2012*) [6],[18],[12],[7], *Phytophthora infestans* (Behnke 1980) [3], *Rhizoctonia solani* (Sharma *et al.* 2009) [25], *Alternaria* (Saxena *et al.* 2008; Lokesha and Naik, 2011) [23], [16].

The concentration of culture filtrate has been reported to be host specific in the selective medium. As studies of the Hartman *et al.* (1984) [11] and Arcioni *et al.* (1987) [1] also suggested that lethal concentrations of culture filtrate of the same host parasite combination varies between 7.5% to 15% depending upon the host genotype.

4.2 Multiplication of selected calli

For selection of the tolerant callus, selected callus was grown on MS medium containing 3 mg/l NAA+ 1 mg/l BAP, which has been demonstrated to be best medium for normal callus multiplication of tomato by Kumar *et al.* (2017) [13]. After 2-3 days subculturing was done on the same medium and when desired callus multiplication was achieved then callus was used for the regeneration of shoots. When calli were cultured on this medium, it turned into light green from pale yellow within seven days. The growth of the selected callus was very slow after selection and though callus has slowly turned green and nodular but regeneration of shoot occurred (Fig. 4). Slow growth of the selected surviving calli on the toxin-free medium was also reported by Gayatari *et al.* (2005) [8] and Thakur *et al.* (2014) [26] and the degree of growth inhibition was found directly proportional to the concentration of culture filtrate added to the medium.

Fig 4: Multiplication of selected callus on MS+ 3.0 mg/l NAA+ 1.0 mg/l BAP after a) 15 days, b) 25 days, c) 35 days

4.3 Attempts to regenerate shoots from the selected and normal callus

For shoot regeneration the calli were cut into small pieces and cultured on MS medium supplemented with different concentrations of BAP and NAA. At low concentrations of BAP i.e from 0.25-0.75 mg/l, callus did not respond for shoot regeneration and turned brown sooner or later. However, when callus cultured on higher concentration of BAP the callus turned into green nodular form and small

Table 2: Effect of different concentrations of BAP and NAA on regeneration of selected callus

BAP and NAA (in mg/l)		Morphogenic reponse	
BAP	NAA	Normal callus	Selected callus
0.25	-	Browning of callus	-
0.50	-	Browning of callus	-
0.75	-	Browning of callus	-
1.00	-	Slightly green	-
1.25	-	Slightly green	-
1.50	-	DG+N	-
1.75	-	DG+N	N+C+DG
2.0	-	N+C+DG	N+DG
2.25	-	N+C+DG+formation of small shoots	N+C+DG
2.50	-	N+C+DG+formation of small shoots	N+C+DG
2.75	-	N+DG+ formation of good shoots	DG+N+formation of small shoots
3.00	-	N+DG+ formation of good shoots	N+C+DG+ formation of small shoots
3.25	-	N+C+DG	N+C+DG
3.50	-	N+C+DG	N+C+DG
2.00	0.10	N+C+DG	-
2.00	0.05	N+C+DG	-
1.00	0.10	N+C+LG	-
1.00	0.05	N+C+LG	-
1.00	0.50	C+LG	-

N: Nodular C: Compact DG: Dark Green LG: Light green

Shoots formation was observed. Slightly raised nodulation and good shoot formation were observed in normal callus on MS medium containing 2.75 and 3.0 mg/l BAP but in case of selected callus slightly raised green colour nodulation and very small or less shoot formation was observed on the same medium (Table 2; Fig. 5).

The shoot regeneration was also not obtained by Sharma *et al.* (2009) [25] in selection of carnation against culture filtrate of *Rhizoctonia solani* Kuhn in the tolerant callus. The gradual decline in the morphogenic potential of selected callus may be due to accumulation of inhibitory substances, decline in metabolism, transport

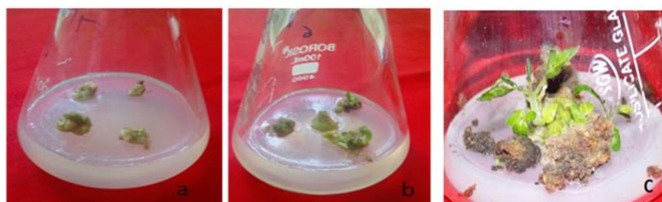

Fig 5: Attempts to regenerate shoots from the callus after a) 15 days, b) 20 days, c) 35 days

And interaction between growth regulators or increase in the number of polyploidy or aneuploid cells in the callus because of prolonged subculturing and exposure to the toxin (Nehra *et al.* 1990) [19].

4.4 Molecular studies for selected and normal callus

ISSR primers were used to understand whether in-vitro generated *Phytophthora* tolerant tomato lines have also been genetically improved during the process of in-vitro selection using selection pressure of the culture filtrate of fungus. ISSR analyses of 5 *Phytophthora* tolerant and control were performed with total of 18 amplified reproducible fragments produced from six ISSR primers. The number of fragments per primer ranged from 2 in primer IISRS-3-F and IISRS-3-L to 4 fragments in primer ISSR-5 and ISSR-7. All the primers were found to be polymorphic and produced different percentages of polymorphism. Primer IISRS-3-F produces 2 fragments, primer IISRS-3-L produces 2 fragments, primer IISRS-3-M produces 3 fragments, primer IISRS-5 produces 4 fragments, primer IISRS-7 produces 4 fragments and primer IISRS-8 produces 3 fragments (Table 3; Fig. 6). It was observed that 50% fragments of IISRS-3-F primer, 50% fragments of IISRS-3-L, 66% fragments of IISRS-3-M, 25% fragments of IISRS-5, 50% fragments of IISRS-7 and 66% fragments of IISRS-8 primer were found to be polymorphic.

This suggests that the selected tolerant calli are different at genomic level from the normal callus and the differences do not only exist at morphological level but they are also present at DNA level and they are genetically improved than the control cell lines.

Table 3: Details of primers used

Primer Name	Primer Sequence	Resolved bands	Scored bands	Monomorphic bands	Polymorphic bands
IISRS-3-F	GACAGACAGACAGACA	2	2	1	1
IISRS-3-L	GACAGACAGACAGACA	2	2	1	1
ISSR-3-M	ACACACACACACACAC	3	3	1	2
ISSR-5	AGAGAGAGAGAGAGAGYC	4	4	3	1
ISSR-7	ACACACACACACACACYC	4	4	2	2
ISSR-8	GAGAGAGAGAGAGAGAYC	3	3	1	2

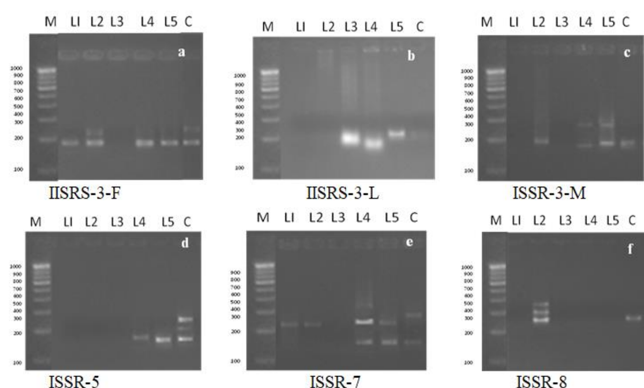

Fig 6 a-f.: Molecular profiles showing polymorphism with six ISSR primers M: 100 bp Marker; L1-L5: Line 1-Line 5; C: Control

DNA fingerprint considered to be a powerful tool for detecting any new mutation may be caused when plant was subjected to stress. The modified polymerase chain reaction (PCR) with single primers of arbitrary nucleotide sequence and requiring no prior sequence information have proved useful in detecting intraspecific polymorphism among organisms as reported by Welsh and McClelland (1990) [28] and Williams *et al.* (1990) [29]. Similarly, Esmaiel *et al.* (2012) [7] used RAPD technique to detect genetic variation at the level of DNA among carnation variants selected for resistance to *Fusarium oxysporum* f. sp. *Dianthi*. From the molecular studies it was observed that the selected (tolerant) calli were different at genomic level from the normal callus.

5. Conclusion

From this study it can be concluded that fungal culture filtrate of *Phytophthora nicotianae* var. *parasitica* was injurious to the tomato callus and decrease in percent survival of callus with increase in concentration of culture filtrate was observed. However the selected calli were obtained at 20.0 % of fungal culture filtrate. In case of calli selected against fungal culture filtrate the regeneration from the callus was also obtained and these shoots can be further use for mass propagation and breeding programme, as changes has been also observed on DNA level in ISSR marker studies. Although it was observed that the selected cells efficiency to regenerate on the normal standardized medium is very low. The above standardized protocol could be further used to obtain disease resistant plants of tomato. Polymorphic bands obtained during the ISSR marker studies can be further eluted, cloned and sequenced.

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7. Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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