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# *In vitro* Selection for Resistance against Charcoal Rot Disease of Soybean [*Glycine max* (L.) Merrill] Caused by *Macrophomina phaseolina* (Tassi) Goid

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## ABSTRACT

**Background:** Soybean is an important oilseed crop. Charcoal rot caused by soil borne polyphagous fungus *Macrophomina phaseolina* in soybean causes about 80 percent yield loss.

**Methods:** Callus and cell suspension cultures derived from immature and mature embryonic axis and cotyledons explants from disease susceptible soybean cultivars (cv JS335 and JS95-60) were treated with purified toxic culture filtrate generated by the fungus *Macrophomina phaseolina* fortified with MS culture medium. A continuous method of four cycles of selection was executed on toxic medium while during discontinuous method, a silence was given after the second and third cycle of selection using non-toxic medium.

**Result:** The discontinuous method appeared to be superior as it permitted the calli to recover their regeneration capability. Continuous exposure to toxic culture filtrate resulted up to about 65-75 percent mortality. A total of four lines of JS335 and nine of JS95-60 were found resistant amongst an array of putative resistant/tolerant lines during $S_1$ generation.

**Key words:** Cell suspension culture, Cell clumps, Embryogenic calli, *In vitro* selection, Toxin culture filtrate.

## INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an economically important dicot legume in the world's oilseed cultivation scenario. Continuous cultivation and simultaneous increase in area of soybean has led to increase in disease (Mishra *et al*. 2020) that ultimately resulted in yield losses. Among biotic stresses, charcoal rot caused by soil borne polyphagous fungus *Macrophomina phaseolina* (Tassi) Goid is a major reason of yield loss in soybean (Yang and Navi, 2005). Disease management practices have not been implemented precisely for controlling the disease (Twizeyimana *et al.* 2012). Therefore, development of charcoal rot resistant varieties is an effective means of controlling the disease.

In soybean, there are several reports on *in vitro* selection technique. In earlier reports, an efficient and reproducible regeneration with similar genotypes have been established previously with diverse explants (Tripathi, 2004; Tiwari and Tripathi, 2005). Sunaryo *et al.* (2017) established *in vitro* selection in soybean against drought. Limited reports are available on development of charcoal rot resistant soybean genotypes/varieties in India. Therefore the present work offers good scope for discovering new genotype (s) that could contribute in enhancing the yield contributing characters as well as tolerant genotypes against charcoal rot in future by applying *in vitro* selection.

## MATERIALS AND METHODS

Two charcoal rot susceptible genotypes *namely*: JS335 and JS-95-60 were used for developing callus and cell suspension cultures and to raise tolerant cell line(s). The seeds were obtained from RAK, College of Agriculture, Sehore, RVSKVV, Gwalior (M.P.). Isolates of *M. phaseolina* were collected from the charcoal rot infected soybean plants found in the experimental field of the College of Agriculture, Jawaharlal Nehru Agricultural University, Jabalpur, MP, India. The standard potato dextrose agar (PDA) medium was used for culturing the *M. phaseolina*. The laboratory experiments were conducted during the years 2018-19 at Department of Plant Molecular Biology and Biotechnology, RVSKVV, Gwalior.

### Isolation of pathogen and pathogenicity test

Soybean plants showing charcoal rot symptoms were collected from the field. The infected root were cut into small bits and washed in running water and surface sterilized with

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one percent of sodium hypochlorite solution for one minute followed by thorough washing for three times with sterile distilled water to remove the traces of sodium hypochlorite and then aseptically transferred to petriplates containing the sterilized PDA medium. The plates were incubated at 28°C±2°C for 4 days. The pure culture of the fungus was maintained by further growing the culture and following hyphal tip culture method under aseptic conditions. Soybean seeds of selected genotypes were autoclaved and inoculated with 3-days old culture of *M. phaseolina.* Inoculated flasks were incubated at 25±10°C for 15 days. Ten seeds of each genotype (JS335 and JS-95-60) were sown in each pot containing *M. phaselina* culture and observed for symptoms development (Fig 1 C-D).

**Preparation of toxic culture filtrate**

For the extraction of toxic culture filtrate, 5 mm discs from 30 days old fungal cultures grown on PDA were transferred to a fresh PDA medium once in every 4 weeks (Fig 1 A). Ten to fifteen pieces of PDA cut from a two-week-old culture of *M. phaseolina* was inoculated in 250 ml Erlenmeyer flask containing 50 ml liquid MS medium (Fig 1B). After two weeks the medium was divided into five equal portions of 10 ml and incubated under dim light at 22°C for six weeks. The suspension was passed through filter paper Number1 (Whatman) and subjected to centrifugation at 10,000xg for 20 min. The ssupernatant was sterilized using nitrocellulose filter (0.22 µm) and was stored at -20°C.

**Establishment of callus and suspension culture**

Callus cultures were established by culturing immature and mature embryonic axis and cotyledons explants on basal MS medium (Murashige and Skoog, 1962) fortified with two different auxins, namely: 2,4-D (2,4 di-chloro phenoxyacetic acid) and NAA (α Napthaleneacetic acid) in varying concentrations and a cytokinin, *viz.* BAP (6 benzylaminopurine) in varying concentrations (as sole). For raising embryogenic cell suspension culture methods described by Tiwari *et al*. (2007) for onion and Uikey *et al*. (2016) for *Rauvolfia serpentina* were followed.

**Determining the $LD_{50}$ *M. phaseolina* toxic culture filtrate**

To discover the selection concentration of phytotoxin, small pieces of calli/cell clumps/embryoids were added with varying concentrations of the toxin. $LD_{50}$ was established with allusion to just about 50% retarded growth of calli/cell clumps/embryoids. Fresh weight and relative growth rates of callus and cell suspension cultures was recorded after 4 weeks of culture after inclusion of 0.0 ml-9.0 ml levels of phytotoxin in the initial culture medium.

***In vitro* selection and regeneration procedures**

Callus and cell suspension cultures were transferred to MS medium fortified with each of 5.0 mg $l^{-1}$ 2, 4-D and NAA, 0.5 $mgl^{-1}$ BA and lethal concentration of toxic culture filtrate (*M. phaseolina*). Final response of cell clumps/embryoids obtained from embryogenic suspension culture to the lethal concentration of toxic culture filtrate and numbers of surviving calli/cell clumps/ embryoid after following continuous and discontinuous method of selection cycle were recorded.

**Regeneration of plants**

Resistant calli/embryoids/cell clumps were transferred into regeneration medium *viz*., MS medium fortified with 0.5 $mgl^{-1}$ each of NAA, BA and Kn, 20.0 $gl^{-1}$ sucrose and 7.5 $gl^{-1}$ agar powder. Cultures were subjected to 25±2°C temperature and photoperiod regimes of 60 m mol $m^{-2}$ $s^{-1}$ luminance provided by cool fluorescent tubes for 16 hrs after 4-5weeks of culture.

**Rooting and hardening**

Regenerants were transferred to MS rooting medium supplemented with 1.0 mg $l^{-1}$ IBA, 15.0 $gl^{-1}$ sucrose and 7.5 $gl^{-1}$ agar powder after 45-50 days. After rooting regenerants were uprooted from cultures and systematically cleaned with running tap water to eradicate the adhering agar, they were planted in 2.5 cm root trainers filled with 1:1:1 sand, soil and FYM sterilized mixture. Root trainers with transplanted plants were subjected to 30±2°C and 65±5% RH in greenhouse for 15-20 days followed by hardening in Net House for 30 days before transplanting to the field.

**Experimental design and data analysis**

Completely Randomized Design was used to find out the significance of different genotypes with two replications. About 100-120 explants per replication were cultured on each media of both genotypes. The data was analyzed as per method suggested by Snedecor and Cochran (1967).

**Molecular confirmation of putative disease resistant plants(s) using RAPD markers**

DNA extraction of both mother genotypes *viz*., JS335 and JS95-60 as well as selected putative charcoal rot resistant plants was performed according to the protocol adopted (Mishra *et al.* 2020). PCR amplifications were done with five random decamer primers *namely* OPC-20, OPA-11, OPB-07, OPC-03 and OPE-11. PCR reaction mixture consisted of: 50 ng genomic DNA, 10 pmol primer, 200 µM of each dNTP and 1 unit of *Taq* DNA polymerase with PCR buffer supplied (TrisHCl, pH 9.0; 15 mM $MgCl_2$). Cycling parameters were: 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C with a final extension time of 5 min at 72°C. Amplicons were separated by electrophoresis on 1.5% agarose gel and visualized under gel documentation system after staining with ethidium bromide.

## RESULTS AND DISCUSSION

*In vitro* selection proffers have gigantic potential for the speedy and comprehensive generation of useful somaclones or mutants for resistance against various biotic and abiotic factors. The present experimental results evince that *in vitro* regeneration of rather difficult species like soybean can be improved upon and can become acquiescent to *in vitro* selection.

**Raising of embryogenic callus and cell suspension cultures**

Significant differences among various explants, different plant growth regulators, concentrations and their interactions with each genotype for callus induction and plantlet regeneration was (Table 1) observed. For callus and cell suspension cultures, MS medium fortified with each of 4.0-5.0 mg.$l^{-1}$ 2, 4-D and NAA in combination with 0.5 mg $l^{-1}$ BA was superior for all culture phases. In subsequent sub culturing use of reduced concentration of 2, 4-D (2.0-3.0

**Table 1:** Initial responses of immature and mature embryonic axis and cotyledons cultured on MS medium supplemented with different plant growth regulators in varying concentrations.

| Culture media | Direct shoot formation | Root for mation | Callus for mation | Non morphogenic friable calli | Callus colour | Callus textureand morphology |
|---|---|---|---|---|---|---|
| MS.5N | - | - | + | - | Yellow greenish | Soft friable |
| MSN | - | + | + | - | Yellow greenish | Soft friable |
| MS2N | - | ++ | ++ | - | Yellow greenish | Soft friable |
| MS3N | - | ++ | ++ | - | Yellow greenish | Soft friable |
| MS4N | - | + | +++ | - | A mixture of yellow, green and whitish calli | Compact |
| MS5N | - | + | +++ | - | A mixture of yellow, green and whitish calli | Compact |
| MS6N | - | + | ++++ | - | Yellow/green | Compact. Large in size |
| MS8N | - | + | ++++ | + | Yellow/green | Compact. Larger in size |
| MS10N | - | + | ++++ | ++ | Dark- yellow | Compact. Largest in size |
| MS.5D | - | + | ++ | + | Yellowish | Soft friable. Callus covered with white boundaries |
| MSD | - | + | ++ | + | Yellowish | Soft friable. Callus covered with white boundaries |
| MS 2D | - | + | +++ | + | Yellowish | Soft friable. Callus covered with white boundaries |
| MS 5 D | - | + | ++++ | + | Yellowish | Soft friable. Callus covered with white boundaries |
| MS10D | - | Rare | ++++ | ++ | Yellowish | Soft friable. Callus covered with white boundaries |
| MS20D | - | Rare | ++++ | +++ | Yellowish/ white | Covered with dense layer of white loose calluses |
| MS30D | - | Very rare | ++++ | +++ | Yellowish/ white | Covered with dense white loose calluses. Comparatively large in size |
| MS40D | - | Very rare | ++++ | +++ | Yellowish/ white | Covered with dense layer of white loose calluses. Largest in size |
| MSB | + | + | + | - | Green | Compact. Covered with white layers of loose calluses |
| MS2B | + | Rare | ++ | - | Green | Compact. Covered with white layers of loose calluses |
| MS3B | ++ | Rare | +++ | - | Green whitish | Compact. Covered with white layers of calluses |
| MS4B | ++ | Very rare | +++ | - | Green | Compact. Covered with white layers of calli |
| MS5B | - | Very rare | +++ | - | Green. After 2–3 weeks of initial culture colour of calli changed in black | Compact. Covered with white layers of calluses |
| | The grading indicated in +, ++, +++ may be explained | | | | | |

Response: Excellent :> 75% (+ + + +) ; High: 50-75% (+ + +); moderate: 25-50% (+ +); low: <25% (+).

mg.l$^{-1}$ 2,4-D) in combination with 4.0-5.0 mgl$^{-1}$ NAA and 0.5 mg.l$^{-1}$ BA supported faster development of embryos and cell clumps. Plantlets in higher frequencies were regenerated on solid MS medium supplemented with each of 0.5 mg.l$^{-1}$ NAA, BA and kinetin, 20 g.l$^{-1}$ sucrose and 7.5g.l$^{-1}$ agar powder. Cultivar JS 95-60 was found more responsive than JS335 for both the culture systems. Immature embryonic axis and cotyledons explants were proved more promising.

**Determination of the $LD_{50}$ of toxic culture filtrate**

At higher toxic culture filtrate concentration (9.0 mll$^{-1}$; Fig 1 O), about more than 75-80% mortality was observed. At minimum level of toxic culture filtrate (1.0 mll$^{-1}$; Fig 1F), significant negative effect on callus growth was not examined. At the level of 7.0 mll$^{-1}$ (Fig 1 L) toxic culture filtrate, about 50-55% callus relative growth was documented and at the level of 8.0 mll$^{-1}$ (Fig 1N) toxic culture filtrate, about 40-45% relative growth of callus was monitored. Thus, $LD_{50}$ of 7.5mll$^{-1}$ (Fig 1M) toxic culture filtrate concentration was used as selection criteria. The control callus (without addition of toxic culture filtrate; 0.0mll$^{-1}$; Fig 1E) grown at the lower concentrations up to 7.0 mll$^{-1}$ (Fig 1 E-L) of toxic culture filtrate looked healthy and survived considerably, whereas, the calli treated with the highest toxic culture filtrate level (Fig 1 O) turned brown in color and died. This regular decrease in the callus survival rate with the toxic culture filtrate in the medium was probably due to the presence of toxic metabolites in the toxic culture filtrate. Similar results were documented by Tripathi *et al.* (2008) in onion against *Alternaria porrii* and Jhankare *et al.* (2011) against *Aternaria alternata*.

***In vitro* selection and regeneration procedures**

During continuous method, soybean genotypes JS335 and JS95-60 showed 36.54 and 36.86 percentage of survival

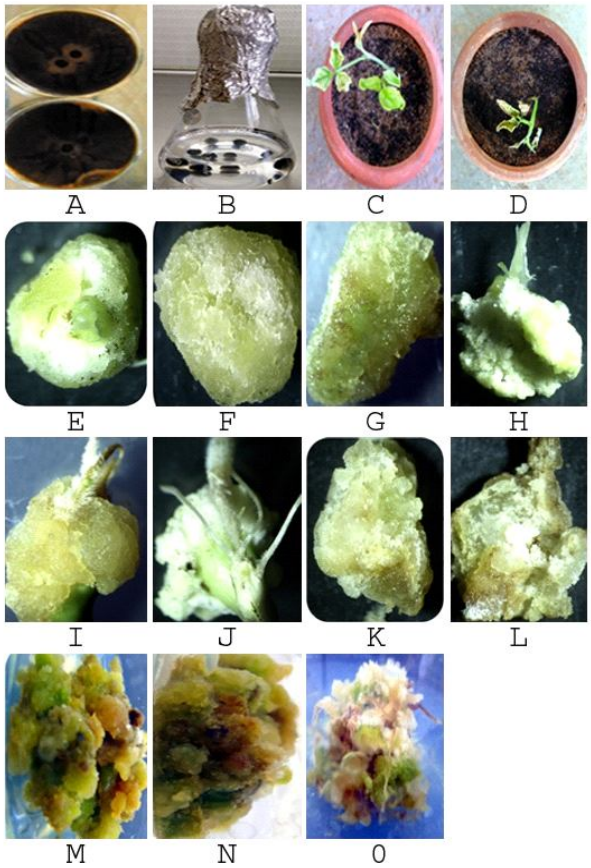


**Fig 1:** *In vitro* selection in soybean against *M. phaseolina* (A-B). Preparation of culture filtrate; (C-D). *In vivo* testing of pathogenicity by inoculation of fungus in soil; Effect of different concentration of toxic culture filtrate on survival of calli on selection medium E.0.0 ml; F.1.0 ml;G.2.0 ml;H.3.0 ml; I. 4.0 ml; J.5.0 ml; K.6.0 ml; L.7.0 ml; M.7.5 ml;N.8.0 ml and O.9.0 ml.

**Table 2:** Comparison of growth rates of mature and immature embryonic axis and cotyledons derived calli from callus and cell suspension cultures of soybean on different levels of toxic culture filtrate in growing medium.

| Concentration of toxic culture filtrate (ml/l) ▼ | Relative growth rate of callus cultures[2] | | Relative growth rate of suspension cultures[3] | |
|---|---|---|---|---|
| **Genotypes ▶** | **JS335** | **JS 95-60** | **JS335** | **JS95-60** |
| 0 | 100.00 ± 0.38 | 100.00 ± 0.40 | 100.00 ± 0.36 | 100.00 ± 0.39 |
| 1.0 | 95.34 ± 0.32 | 97.91 ± 0.30 | 90.52 ± 0.34 | 98.78 ± 0.34 |
| 2.0 | 85.13 ± 0.26 | 88.48 ± 0.28 | 89.86 ± 0.30 | 85.86 ± 0.32 |
| 3.0 | 73.52 ± 0.24 | 75.68 ± 0.25 | 71.52 ± 0.26 | 76.34 ± 0.29 |
| 4.0 | 65.13 ± 0.26 | 68.48 ± 0.28 | 69.86 ± 0.30 | 75.86 ± 0.32 |
| 5.0 | 63.52 ± 0.24 | 65.68 ± 0.25 | 61.52 ± 0.26 | 66.34 ± 0.29 |
| 6.0 | 55.13 ± 0.26 | 58.48 ± 0.28 | 59.86 ± 0.30 | 64.86 ± 0.32 |
| 7.0 | 53.12 ± 0.22 | 54.19 ± 0.24 | 57.18 ± 0.24 | 54.40 ± 0.26 |
| 8.0 | 41.98 ± 0.20 | 43.78. ± 0.22 | 42.56 ± 0.22 | 45.84 ± 0.20 |
| 9.0 | 19.75 ± 0.16 | 21.54 ± 0.18 | 22.90 ± 0.16 | 24.62 ± 0.18 |

[1]Mean was obtained from weights of five inoculums/ treatments after 4 weeks of culture.

[2]&[3]Callus and cell suspension cultures were cultured on MS medium fortified with each of 5.0 mg l$^{-1}$ 2, 4-D and NAA, 0.5 mgl$^{-1}$ BA and different levels of toxic culture filtrate (*M. phaseolina*).

Callus and cell growth appeared average fresh weight (g) of 1.0 g calli.

**Table 3:** Response of immature and mature embryonic axis and cotyledons derived calli of soybean to the lethal concentration of toxic culture filtrate of *M. phaseolina.*

| Cultivar ▼ Selection Cycle ▶ | Number of calli | Number of surviving calli after 4 selection cycles | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| **Continuous method** | | | | | |
| JS335 | 249 | 91(36. 54%) | 83(33.33%) | 79(31.72%) | 72(28.91%) |
| JS95-60 | 274 | 101(36.86%) | 95(34.67%) | 84(30.65%) | 77(28.10%) |
| **Discontinuous method** | | | | | |
| JS335 | 299 | 116(38.79%) | - | - | 105(35.11%) |
| JS95-60 | 321 | 133(41.43%) | - | - | 121(37.69%) |

**Table 4:** Response of cell clumps/embryoids obtained from embryogenic cell suspension cultures of soybean to the lethal concentration of toxic culture filtrate of *M. phaseolina.*

| Cultivar Selection Cycle | Number clumps | Number of surviving calli after 4 selection cycles | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| **Continuous method** | | | | | |
| JS335 | 387 | 135(34. 88%) | 124(32.04%) | 113(29.19%) | 104(26.87%) |
| JS95-60 | 423 | 149(35.22%) | 134(31.67%) | 122 (28. 84%) | 118(27.89%) |
| **Discontinuous method** | | | | | |
| JS335 | 399 | 146(36.59%) | - | - | 134(33. 58%) |
| JS95-60 | 412 | 153(37.13%) | - | - | 141(34.22%) |

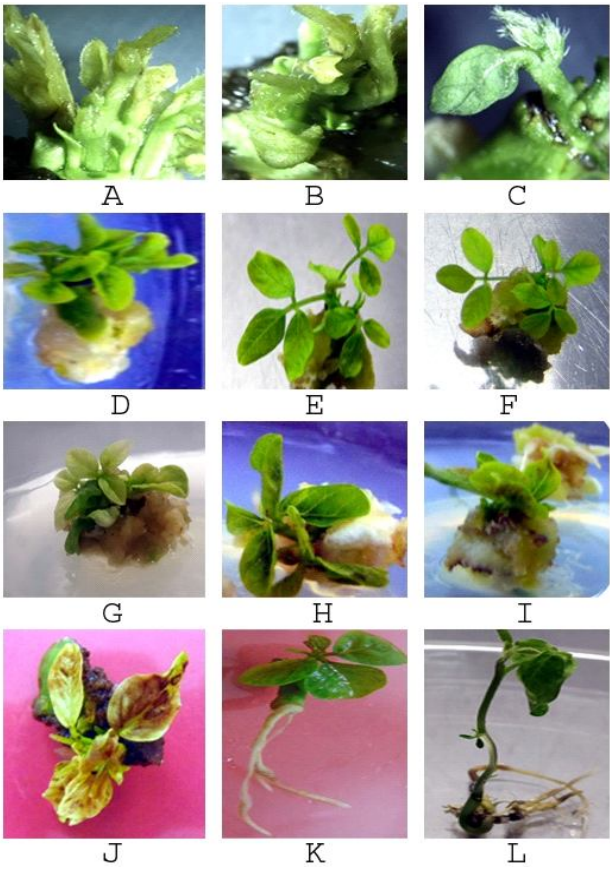


**Fig 2:** Effect of different concentration of toxic culture filtrate on regeneration of plantlets obtained from tolerant calli/ in soybean A.1.0 ml;B.2.0 ml; C.3.0 ml; D.4.0 ml; E.5.0 ml;F.6.0 ml;G.7.0 ml;H.7.5 ml; I. 8.0 ml; J.9.0 ml and K-L. *In vitro* rooting.

rates respectively after first cycle of selection (Table 3). However, during the second cycle about 3-4% of the calli still died but thereafter calli subjected to further selections exhibited insensitivity to medium with toxic culture filtrate, since smaller number of calli died (2-3%). Selection by continuous method resulted in 28.91% callus survival for genotype JS335 and 28.10% for genotype JS95-60 after four cycles of selections. During discontinuous method, 35.11% calli of genotype JS335 and 37.69 % calli of genotype JS95-60 survived.

With the adoption of continuous method with cell suspension cultures, the selections resulted in 34.88% survival for genotype JS335 and 35.22% for genotype JS95-60 after the first cycle of selection (Table 4). About, 3-4% of the cell clumps still died during the second cycle before acquiring insensitivity during the third cycle. Thereafter only few embryoids/cell clumps (2-3%) died due to toxic culture filtrate present in medium during the four$^{th}$ round of selection. Whereas, in selection by discontinuous method, 33.58% and 34.22% embryoids/ cell clumps of genotypes JS335 and JS95-60 respectively survived. In the present investigation, first cycle selection resulted in about 62-72 percent callus mortality in both the genotypes. Discontinuous method exhibited improvement of higher regeneration aptitude over the continuous method.

### Regeneration of plants

A total of 11.32% (24) plantlets of genotype JS335 and 12.50% (33) of genotype JS95-60 were regenerated from tolerant calli/embryoids/cell clumps after transferring them in regeneration medium which was MS basal medium supplemented with 0.5 mgl$^{-1}$ each of NAA, BA and Kn, 20.0 gl$^{-1}$ sucrose and 7.5 gl$^{-1}$ agar powder (Fig 2A-J). They rooted

subsequently after transferring in rooting medium (Fig 2K-L). Putative disease resistant regenerants were transferred in greenhouse (Fig 3 A-C) followed by hardening in Poly House (Fig 3D).

*In vivo* testing of 24 regenerated plants of JS335 to the pathogen revealed four resistant plants, eight intermediately tolerant plants with very few disease symptoms and twelve susceptible plants. Similarly, out of 33 regenerated plants of JS95-60, nine were resistant to the pathogen, 14 intermediate and 10 susceptible (Fig 2H-L & Fig 3 A-D). Exposure and selection of callus cultures to toxin resulted in plants that were tolerant to the pathogen. Variation in reaction to the pathogen observed indicates that the tolerance to *M. phaseolina* in soybean is possibly due to changes in nuclear genome (Breyer *et al.* 2014).

**Molecular confirmation of putative disease resistant plant (s)**

Among all the applied RAPD markers, OPE-11 was found to be able to produce polymorphic bands with template DNA of selected putative resistant plants produced after taking JS335 as mother genotype (Fig 4a). Primer OPE-11

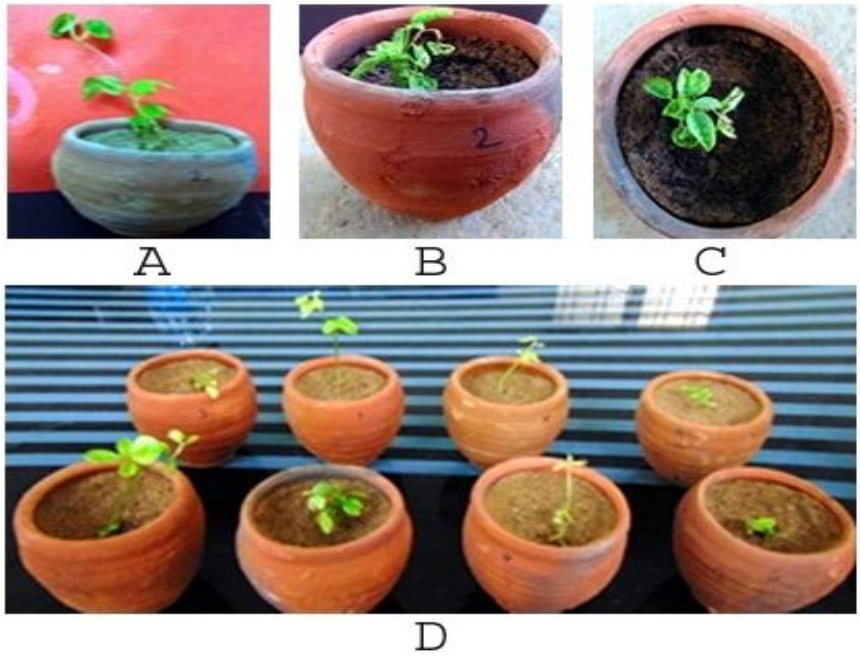


**Fig 3:** Hardening of putative charcoal resistant/tolerant plants of soybean: A-C. Regenerants transferred in Green House and D. Regenerants transferred in Net House for hardening.

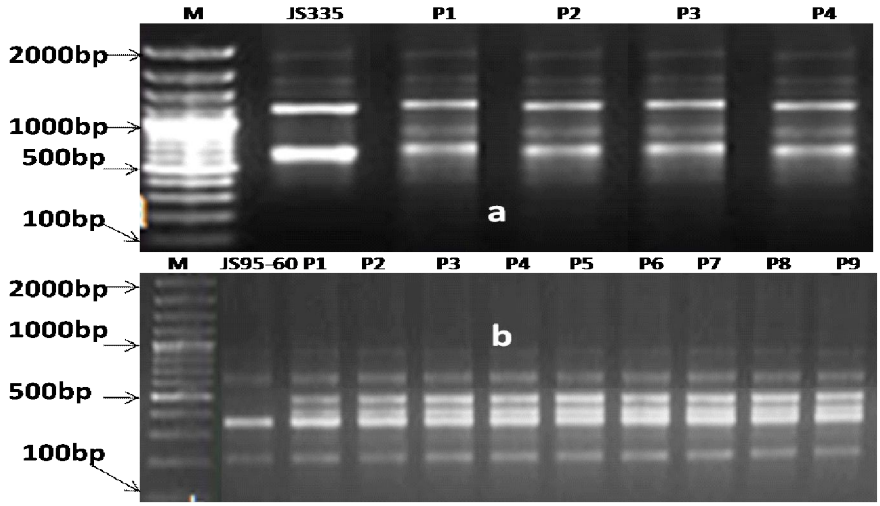


**Fig 4:** Electrophoretic banding pattern of a. OPE-11 and b. OPB-07 RAPD primers in selected putative ccharcoal rot resistant plants with their mother plants JS335 and JS95-60.

amplified a polymorphic band sized 800bp with all four putative resistant plants selected from JS335 however, the absence of this polymorphic band in donor genotype confirms the presence of variability between donor as well as selected putative charcoal rot resistant plants. Molecular variability between donor genotype JS95-60 and selected plants found to be putative resistant against charcoal rot after field evaluation were confirmed with the primer OPB-07 (Fig 4b). Because, primer OPB-07 produced polymorphic amplicons (400bp and 500bp) with the putative resistant plants selected from JS95-60. However, both of these amplicons were absent in their mother plant JS95-60. Similarly, RAPD markers were used by Boscherini *et al.* (1999) for the confirmation of the salt tolerant somaclone of tomato plants.

This approach of generating disease resistance raises question whether the trait acquired is due to the mutation or somaclonal variation. Selection of disease resistant plants without other apparent mutations has been accomplished. Previous reports are in agreement with our findings (Tripathi *et al*. 2008; Jhankare *et al*. 2011).

## CONCLUSION

Immature embryonic axis and cotyledon explant was proved more responsive than mature ones for establishment of callus and cell suspension cultures. MS medium fortified with each of 4.0-5.0 mg.$l^{-1}$ 2,4-D and NAA in combination with 0.5 mg $l^{-1}$ BA was superior for the most of the *in vitro* culture phases. $LD_{50}$ of 7.5ml$l^{-1}$ toxic culture filtrate concentration was used for selection criteria. The discontinuous method appeared to be superior than continuous. The technique has been proved useful in identifying resistant plants for corresponding pathogens using culture filtrate approach. Hence, it could be a method of preference for developing fungal disease resistant genotypes in soybean.

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