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Leaf explant derived callus inducing cell line selection in *Punica granatum* L. (Daru) against *Xanthomonas axonopodis pv. punicae*

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ABSTRACT: *Wild pomegranate (*Punica granatum *L.(Daru)) is prone to many disease but bacterial blight is the main disease affecting it. Surface sterilized mature leaf was studied for indirect regeneration to production of* in vitro *cell lines. Callus induction from mature leaf explants was done on medium containing NAA (2.0 mg/l) and BAP (4.0 mg/l). Shoot regeneration done on medium containing BAP (2.0 mg/l) and Kinetin (0.5 mg/l). Rooting of* in vitro shoots *was done on half strength MS medium supplemented with 0.04% charcoal. Culture filtrate of* Xanthomonas axonopodis pv. punicae *used as selective agent for cell line selection. Resistant lines were selected at 30 per cent level of culture filtrate. From selected calli microshoots were regenerated and these microshoots were further tested under* in vitro *conditions. After testing five resistant plantlets were obtained. Rooting of selected microshoots done on half strength MS medium with 400 mg/l activated charcoal.*

Key words*: Punica granatum L., Cell line selection, Culture filtrate, Xanthomonas axonopodis pv. punicae, in vitro selection, Bacterial blight.*

INTRODUCTION

Wild pomegranate, *Punica granatum* L. (Daru) belongs to family Punicaceae. The pomegranate tree is native from Iran to the Himalayas in Northern India and has been cultivated since ancient times through out the Mediterranean region in Asia, Africa and Europe. The tree is also valued for its pharmaceutical and ornamental properties [20]. Daru is a kind of pomegranate which grows wild in forests and wasteland throughout the Mid-Himalayan region in very large number. The best quality of fruit is obtained in semi-arid region where the temperature during the ripening season is relatively high [27]. Pomegranate seed oil contains punicic acid (65.3%), palmitic acid (4.8%), stearic acid (2.3%), oleic acid (6.3%), and linoleic acid (6.6%) [1]. The higher phenolic content of the peel yields extracts for use in dietary supplements and food preservatives [12]. The bark of the pomegranate tree are used as a traditional remedy against diarrhea, dysentery, and intestinal parasites [15]. The bark is also used in tanning industry [22]. Properties of aril and husk such as antitoxidant, anti-inflammatory and antisclerotic against some diseases (osteoarthiritis, prostrate cancer, heart diseases, HIV-I), have been reported [14,21] and [30]. Leaves, seeds and bark have displayed hypotensive, antispasmodic and antihelminthic activity in bioassay. The fruit is a good source of sugar, vitamin C and fair source of Iron [23].

Pomegranate regarded as a "vital cash crop" is mainly affected by bacterial disease popularly known as 'bacterial blight'. It is one of the most devastating disease of pomegranate occurring in major pomegranate growing states of India. It was first reported in India from Delhi [5]. The disease is characterized by the appearance of one to several small water soaked, dark coloured irregular spots on leaves. [10] stated that disease started as brown to black spots around the nodes. In advanced stages of nodal infection, girdling and cracking of nodes occurred which finally lead to breakdown of branches. [32] reported typical black symptoms on flowers, leaves and fruits of pomegranate incited by *Xanthomonas axonopodis pv. punicae.*

Xanthomonas campestris pv. punicae was identified as the pathogen causing bacterial blight of pomegranate [6]. [10] identify that pathogen causing bacterial blight of the pomegranate is *Xanthomonas campestris pv. punicae* [6]. Later on according to [34]

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name of *Xanthomonas campestris pv. punicae* has been changed to *Xanthomonas axonopodis pv. punicae* [6,33].

[28] reported for the first time that bacterial blight is a severe problem in Himachal Pradesh. There occur 90 to 100 per cent losses due to bacterial blight and incident of bacterial blight ranged between 66 to 90 %. A survey of pomegranate growing areas conducted during June to September 2011 in Solan district of H.P. revealed that biggest threat to pomegranate cultivation is posed by *Xanthomonas axonopodis pv puinicae* causing leaf and fruit spots and subsequent fruit rotting beside causing stem and twig canker. In Kullu and Mandi district of Himachal Pradesh some cases of bacterial blight disease also reported [11].

It is a national concern and has been observed damaging the pomegranate crop in moderate to severe proportion resulting in enormous crop loss to farmers. Therefore, development of resistant varieties is very important to prevent crop loss. 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 Genetic variation is an alternative to conventional breeding, for obtaining somaclonal variants generated by tissue culture techniques. For screening of target characters it is essential to have efficient selection agents [13]. Pathogen toxins are used as selective agent at the tissue culture step might be a source of variability that can lead to selection of individuals with suitable levels of resistance to toxin and/or to pathogen [2]. This approach possess immense potential for easy generation of useful somaclones for resistance to various abiotic and biotic stresses. During present investigation, leaf explants derived callus induced cell line selection was done using bacterial culture filtrate for development of bacterial blight resistant wild pomegranate.

MATERIAL AND METHODS

Plant material

Mature leaves were collected from wild pomegranate (Daru) tree growing in Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). Leaves were thoroughly washed under running tap water for half an hour followed by five minutes treatment with five per cent (v/v) aqueous solution of teepol and rinsed four times with distilled water. Leaves were taken as explants for callus induction. Leaves were surface sterilized with 0.2% bavistin (5 to 15 minute) and 0.5 per cent (v/v) sodium hypochlorite solution (4% chlorine available) for 2.5 and 5 minutes on surface sterilization of leaves. There were six treatments, each treatment having 27 leaf explants replicated thrice following completely randomized design.

Callus induction and shoot regeneration

Mature leaves were used as explants for callus induction and regeneration of plantlet. The explants were excised and cultured on MS medium supplemented with different concentrations of combination of NAA (2.0-5.0 mg/l) and BAP (1.0-2.0 mg/l). The explants were evaluated for per cent callus induction, type of callus, colour of callus, growth of callus. For every combination 20 explants were taken and each experiment was repeated thrice, following completely randomized design.. *The calli were divided into small pieces and cultured on MS medium supplemented with various combinations and concentrations of growth regulators viz. BAP, Kinetin and GA_3 to optimize the culture medium for shoot regeneration. The cultures were maintained at 24±2 °C under 16 hour photoperiod. The experiment consisted of 20 explants in each treatment, which were replicated thrice following completely randomized design. Observations were recorded after four week of incubation for per cent shoot bud induction, average number of shoots per callus, average shoot length (cm) for each treatment.*

Shoot multiplication

The regenerated shoots were then multiplied on already standardized medium (MS basal + 2.0 mg/l BAP + 0.5 mg/l Kinetin + 0.5 mg GA_3) for multiplication of shoots.

Pathogen isolation

The different parts of Daru plant showing characteristic symptoms of bacterial infection were collected from infected trees. The bacteria *(Xanthomonas axonopodis pv. punicae)* was isolated from these infected parts and identified on the basis of cultural and morphological characters. The cultures were multiplied and maintained in petridishes containing nutrient agar medium. After inoculation, culture was incubated at 37°C for 2-3 days till uniform growth was obtained. Petriplates were covered properly and after that stored at low temperature (4°C) to stop further growth.

Testing the bacterial pathogenicity

For testing the pathogenecity, fresh healthy leaves of Daru were taken. Leaves were washed under running

tap water, then treated with 70 per cent ethanol. Injuries were made by pricking the leaves with sharp blade and kept incubated in petriplates containing bacterial suspension, autoclaved distilled water and nutrient broth were used as control. Three leaves in each petriplate were taken, both control and treated leaves were incubated in culture room.

Bacterial culture filtrate extraction

The pathogen was inoculated in 500 ml liquid broth and after that incubated at 37°C. After 15-30 days, when all bacterial toxins were released in medium, the bacterial cultures were used for preparation of the culture filtrate which was separated under aseptic conditions in the laminar air flow cabinet. The filtration was done in following three phases. i) First of all, coarse filtration through sterilized ordinary filter paper. ii) Centrifugation at 10,000 rpm for 15 minutes. Then filtration was done through whatman filter paper number 42. iii) Final filtration done by passing the culture filtrate through sterile nylon membrane filter (0.22 μm) with the help of sterile syringe.

After preparation of culture filtrate, it was kept for 48 hours in the culture room to allow the growth of bacteria if there were any in the culture filtrate. After this period, when no growth was observed the filtrate was stored at 4°C and used further for preparation of selective media. For testing the toxicity of the culture filtrate small pieces of Punica callus were incubated in 100 per cent culture filtrate of Xanthomonas axonopodis pv. punicae for 48 hours.

Preparation of selective medium and calli culturing

The medium used for selection was prepared by mixing the filter sterilized culture filtrate of pathogen with sterilized molten MS medium supplemented with 2.0 mg/l BAP and 4.0 mg/l NAA so as to obtain v/v concentration of 0, 10, 20, 30, 40 and 50 per cent, respectively. The medium was thoroughly mixed with culture filtrate and poured in sterile petriplates under laminar air flow cabinet. After this, the petriplates were kept for a week to ensure that the medium was uncontaminated. In vitro grown callus was cut into small pieces of about 20 mg and then inoculated onto selective media of different concentrations under the laminar air flow cabinet. Flasks were subsequently sealed with parafilm and incubated in the culture room 16 hr photoperiod and at temperature of 25±2°C. The growth of the cells was monitored by their ability to divide and form colonies. Survival per centage of callus on different selective media was also noted.

Selection of resistant cell lines

The highest concentration of culture filtrate at which calli survived was recorded. The green/pale yellow colour of callus depicted that cells were alive whereas dark brown colour indicated dead cells. Multiplication of the selected calli that survived two cycles of selection was done on medium devoid of culture filtrate.

Shoot regeneration from the selected cell lines and there multiplication

Selected callus was transferred to medium standardized earlier for shoot regeneration . The regenerated shoots were then multiplied on the shoot multiplication medium.

Testing of resistance

Selected shoots were tested for resistance against bacterial blight under *in vitro* conditions. For testing shoots were taken selected from each treatment and control shoots (unselected) were inoculated with the bacterial suspension and then transferred to multiplication medium. The inoculated shoots were observed daily and observations regarding development of symptoms were recorded.

In vitro rooting and hardening

Shoots of 2.0-3.0 cm in length were excised at different subculture stages. These shoots were then transferred to half strength MS medium with 0.04% activated charcoal for root induction to get a complete plant. Complete plantlet was then hardened in pots containing sterilized sand.

RESULTS

Callus induction and shoot regeneration

Establishment of callus cultures using leaf as explants

Treatment T_6 consisting of 0.2% bavistin treatment for 15 minutes and 0.5% sodium hypochloride treatment for 5 minutes showed maximum percent uncontaminated culture of 88.89% after 6 weeks of incubation but percent survival of explants was only 37.03%. However treatment T_4 consisting of 0.2% bavistin treatment for 10 minutes and 0.5% sodium hypochloride treatment for 5.0 minutes resulted in 80.25% uncontaminated cultures with 88.88% of survival of explants. This implies that with increase in time duration of treatment the survival percent of explants decreases.

No callus formation was observed in control medium devoid of any plant growth regulators, however among the different concentrations of BAP and NAA, treatment E_7 comprising (2.0 mg/l) BAP and (4.0 mg/l) NAA showed the highest percent of callus induction (71.67%) in comparision to other treatments. Callus formed is compact and green in colour in all cases. Fastest callus growth occurred in E_7 treatment. Treatment E_3 comprising of 2.0 mg/l BAP and 2.0 mg/l NAA showed minimum per cent callus induction (10.00%) with slowest growth rate. E_5, E_6 and E_8 showed moderate rate of growth. While no callus induction was observed on E_2 and E_9 treatment (Table 1).

It was noticed that the treatment E_7 consisting of 2.0 mg/l BAP and 4.0 mg/l NAA showed compact green callus with highest growth (Fig.1b). Hence treatment E_7 was statistically significant and considered best for callus induction and proliferation from leaf explant. [25] found that addition of NAA in combination with BA to MS medium was essential to induce callus. [3] in pomegranate (cv. Ganesh) also reported best callus induction on MS medium supplemented with 4 ppm NAA + 2 ppm BAP + coconut water using cotyledon, hypocotyl, and leaf as explants. [18] reported callus induction in pomegranate from anthers on nutrient media containing BA and NAA.

Callus differentiation and shoot regeneration

No shoot bud induction from callus was observed in control medium (G_1) devoid of plant growth regulators. However, maximum frequency of shoot regeneration (48.33 per cent) was observed on treatment (G_3) comprising of BAP (2.0 mg/l), Kinetin (0.5 mg/l) from leaf derived callus which is at par with treatment G_{12} and G_{16}. It was noticed that highest number of shoots (6.66) per callus clump as well as largest shoot length (1.83 cm) was observed on same (G_3) treatment as compared to all other treatments. Minimum percent shoot regeneration (15.00 per cent) from leaf derived callus was observed in G_7 treatment comprising of BAP (2.0 mg/l) and Kinetin (1.0 mg/l), which is at par with treatment G_8, G_9, G_{10} and G_{11} (Table 2). Cytokinins are very effective in promoting direct or indirect shoot initiation. A high frequency of shoot organogenesis from leaf derived callus of pomegranate was achieved on MS medium supplemented with 1.0 mg/l BAP [7]. High frequency of shoot differentiation was obtained on MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/lNAA, in *Punica granatum* L. cv. Ganesh ([19]. A high frequency of shoot organogenesis was obtained when explants were incubated on MS medium supplemented with 8 µM BA, 6 µM NAA and 6 µM GA_3 [9] (Fig. 1c).

Shoot multiplication

For shoot multiplication, the individual shoots from the shoot clumps measuring 1.5-2.0 cm were separated and cultured on the already standardized shoot multiplication medium (MS medium containing BAP (2.0 mg/l), Kinetin (0.5 mg/l) and GA--$_3$ (0.5 mg/l)) till sufficient rate of multiplication was achieved. After subculturing at an interval of 4 weeks, it was observed that the rate of shoot multiplication increased subsequently with increase in subculturing passage (Fig. 1d). Role of cytokinins in *in vitro* shoot multiplication was reported by several workers [824,29].

In vitro selection

The calli of *Punica* cut into small pieces of about 20 mg each and were subjected to cell selection experiment. Hundred per cent survival of inoculated calli at zero per cent level of culture filtrate (control). Further increase in the concentration of culture filtrate decreased the per cent survival of *Punica* calli. Survival rate was zero per cent and the calli turned brown and became dead at 40 per cent level of culture filtrate. The cell selection was carried out at 30 per cent of culture filtrate concentration where cell survival was fifteen per cent only (Table 3). Therefore, the treatment with 30 per cent bacterial culture filtrate is optimum for selecting resistant cell lines. *In vitro* cell selection of apple rootstock MM106 against *Phytophthora cactorum* was reported by [4] at 20.0 per cent concentration of culture filtrate for selection of calli. [31] reported *in vitro* selection and regeneration of carnation plants resistant to *Fusarium oxysporum* f. sp. *dianthi* at 15 per cent culture filtrate. The surviving calli were subjected to 2 cycles of selection. [17] reported *in vitro* selection of carnation *Dianthus caryophyllus* callus culture tolerant to *Alternaria dianthi* at 15.0 per cent selective dose of culture filtrate where survival rate of calli was 11.67 per cent.

Surviving calli were further subcultured on the same concentration of bacterial culture filtrate. The selected callus subcultured on MS medium supplemented with 2.0 mg/l BAP, 4.0 mg/l NAA and 30 per cent BCF (Bacterial Culture Filtrate) for three times at the interval of four weeks. After two cycles of selection the callus was cultured on callus multiplication medium (MS medium + 2.0 mg/l BA

+ 4.0 mg/l NAA) for proliferation of callus. With increasing passage of subculturing there was increase in fresh weight of callus and highest fresh weight of callus (1.88 g) was observed after third subculture.

In vitro shoot regeneration from resistant calli

The selective calli was cultured on shoot regeneration medium (MS medium + BAP (2.0 mg/l) + Kinetin (1.5 mg/l) and GA_3 (3.0 mg/l)) without culture filtrate. The cultures were incubated at 26±2 °C and 16 hour photoperiod. No shoot regeneration was observed after first and second subculturing. However, after third subculturing 55.55per cent shoot regeneration from selected calli was observed with three average number of shoots per callus clump and 0.71cm shoot length.

In vitro testing of plantlets regenerated from resistant calli

Plantlets regenerated from selected calli were tested *in vitro* by treating them with suspension of pure cultures of *Xanthomonas axonopodis pv. punicae*. Suspension of pure culture was prepared by dissolving 1 mm^2 bit of *Xanthomonas axonopodis pv, punicae* in 50 ml of distilled water. Ten control microshoots and 10 microshoots regenerated from selected calli were inoculated in suspension. Shoots were then cultured on shoot multiplication medium. Whole experiment was carried out under asceptic conditions. Observations were taken after 3-4 days interval. After one week symptoms appeared in all 10 control shoots, while only slight symptoms were observed in five shoots out of 10 shoots. No symptom was appeared in five shoots out of 10 shoots regenerated from selected calli used in the experiment. Which were expected to be resistant shoots (Fig. 2a,b). [26] reported *in vitro* selection of sugarcane genotypes CoJ 88 and CoJ 64 against *Colletotrichum falcatum* causing red rot of sugarcane while the somaclones generated from resistant/ tolerant calli exhibited better resistance than the parental genotypes when tested *in vivo.* [31] observed considerable resistance against the pathogen *Fusarium oxysporum* f. sp. *dianthi* in the field in *in vitro* selected carnation (*Dianthus caryophyllus* L.) plants resistant to culture filtrate of fungus.

In vitro rooting and hardening

For root regeneration, the selected *in vitro* resistant shoots were transferred to rooting media containing half strength MS media supplemented with 0.04% activated charcoal. Root induction within 10-15 days in culture and profuse rooting was observed within 4 weeks (Fig. 2c,d). Complete plantlet get developed and it was hardened in pots containing sterilized sand (Fig. 2e). Rooting on MS medium containing 500 mg/ l of activated charcoal was observed in shoots of *Punica granatum* L.cv. Kandhari Kabuli [25].

CONCLUSION

The use of *in vitro* technique in combination with induced mutations can speed up the breeding programmes for generation of variability through selection and multiplication of desired genotypes [16]. *In vitro* selection for disease resistance presents an excellent opportunity to assess the direct application of tissue culture to crop improvement. Genetic variation can be enhanced by cell and tissue culture (somaclonal variation technology) and may possibly lead to new source of resistance, and *in vitro* selection procedures may increase the efficiency of selection. The toxic metabolites present in the culture filtrate play role in developing resistance against pathogen and may be an effective screening agent in cell selection program.

Table 1
Effect of different concentrations of BAP in combination with NAA on per cent callus induction from mature leaf explants of 10 year old tree after 4 weeks of incubation

Treatment	*BAP (mg/l)*	*NAA (mg/l)*	*Per cent Callus induction*	*Type*	*Colour*	*Growth*
E_1	0.00	0.00	0.00(0.00)	-	-	-
E_2	1.00	2.00	0.00(0.00)	-	-	-
E_3	2.00	2.00	10.00(18.04)	C	G	+
E_4	1.00	3.00	13.33(21.32)	C	G	+
E_5	2.00	3.00	20.00(26.44)	C	G	++
E_6	1.00	4.00	18.33(25.29)	C	G	++
E_7	2.00	4.00	71.67(57.83)	C	G	+++
E_8	1.00	5.00	26.67(30.93)	C	G	++
E_9	2.00	5.00	0.00(0.00)	-	-	-
$CD_{0.05}$			4.16			
SE±			1.36			

* Figures in parentheses are arc sine transformed values

C : Compact G : Green +: Slow growth
+++ : Fast growth ++ : Moderate growth - : No growth

Table 2
Effect of different concentrations of BAP and Kinetin along with GA_3 for shoot regeneration from leaf derived callus after 4 weeks of incubation

Treatment	*BAP (mg/l)*	*Kinetin (mg/l)*	*GA3 (mg/l)*	*Per cent Shoot regeneration in leaf callus*	*Average No. of shoots per leaf callus*	*Shoot Length (cm)*
G_1	0.00	0.00	0.00	0.00(0.00)	0.00	0
G_2	1.0	0.5	-	26.67(31.05)	2.66	0.44
G_3	2.0	0.5	-	48.33(33.14)	6.66	1.83
G_4	3.0	0.5	-	25.00(29.91)	5.00	1.00
G_5	4.0	0.5	-	30.00(44.02)	5.66	0.94
G_6	1.0	1.0	-	40.00(39.19)	5.33	0.74
G_7	2.0	1.0	-	15.00(22.58)	5.66	0.40
G_8	3.0	1.0	-	16.67(23.73)	5.00	0.52
G_9	4.0	1.0	-	15.76(22.58)	4.66	0.42
G_{10}	1.0	0.5	1.0	16.67(23.15)	5.66	0.45
G_{11}	2.0	0.5	1.0	16.67(23.15)	6.00	0.89
G_{12}	3.0	0.5	1.0	45.00(42.10)	6.33	0.43
G_{13}	4.0	0.5	1.0	38.33(38.20)	5.00	0.47
G_{14}	1.0	1.0	2.0	35.00(36.22)	4.33	0.39
G_{15}	2.0	1.0	2.0	40.00(39.19)	5.66	0.46
G_{16}	3.0	1.0	2.0	45.00(42.10)	6.00	0.89
G_{17}	4.0	1.0	2.0	36.67(37.18)	5.33	0.71
$CD_{0.05}$				7.36	2.78	0.21
S.E.±				**2.55**	**0.96**	**0.07**

*Figures in parentheses are arc sine transformed values

Table 3
Cell line selection of *Punica granatum pv. punicae* against *Xanthomonas axonopodis pv. punicae* culture filtrate after 4 weeks of inoculation

Treatment	*Concentration of culture filtrate (%)*	*Mean per cent survival of cell lines*
X_1	Control	100.00(90.00)
X_2	10	60.00(48.96)
X_3	20	50.00(47.10)
X_4	30	15.00(9.99)
X_5	40	0.00(0.00)
$CD_{0.05}$		6.44
SE±		**2.38**

* Figures in parentheses are arc sine transformed values

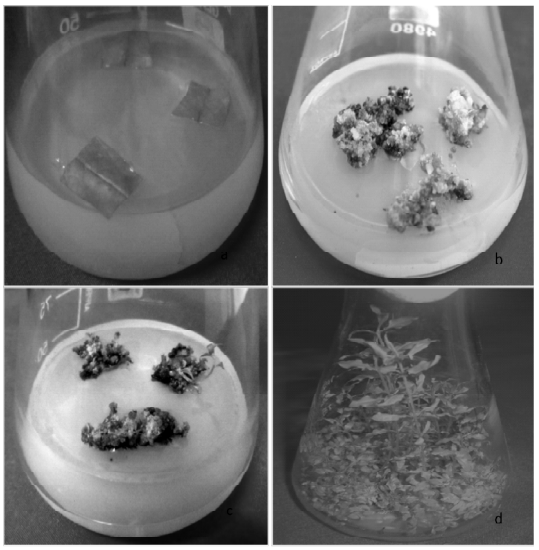

Figure 1: a. Mature leaf explant incubated on medium for callus induction b. Callus induction from leaf explants c. *In vitro* shoot bud induction from leaf derived callus d. *In vitro* shoot proliferation

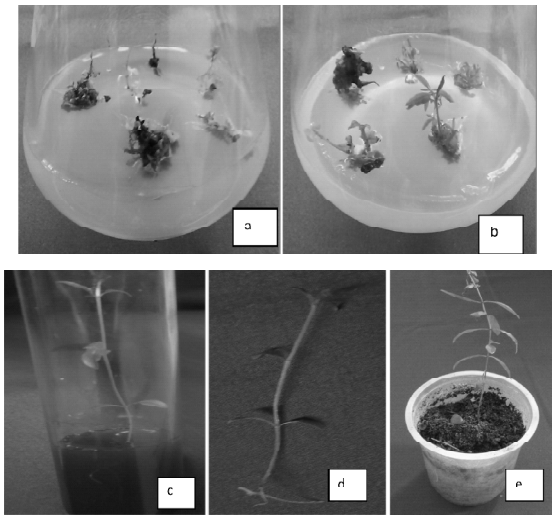

Figure 2: *In vitro* testing of microshoots inoculated with bacterial suspension a. Control microshoots b. Resistant microshoots c. *In vitro* rooting of selected shoot on half strength MS basal medium + 0.04% activated charcoal d. *In vitro* raised plantlet (Resistant).e. Hardened plant

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