***In vitro* selection of calli of *Citrus jambhiri* Lush. for tolerance to culture filtrate of *Phytophthora parasitica* and their regeneration**

Savita · Gurdeep Singh Virk · Avinash Nagpal

Published online: 14 January 2011

© Prof. H.S. Srivastava Foundation for Science and Society 2011

Abstract The present study deals with the selection of *Phytophthora* tolerant lines of *Citrus jambhiri* and their regeneration. Cotyledon derived calli were cultured on selective MS medium supplemented with 5–100 % of culture filtrate (CF), to estimate the critical concentration of the selective agent. The survived calli under stress were subcultured for mass propagation for 20 days on callus multiplication medium (2,4-D 2 mg/L + BA 0.75 mg/L) without culture filtrate. After multiplication, these calli were further exposed to other cycles of selection, which contained the same and the 3 steps higher concentrations of the selective agent (CF) and this procedure was repeated several times until the selection regime completed. The selected tolerant calli were transferred to regeneration medium (MS medium supplemented with 3 mg/L of BA and same concentration of culture filtrate on which the calli were selected). Regenerated shoots were transferred to rooting medium ($\frac{1}{2}$ strength MS medium supplemented with 0.5 mg/L of NAA). Under *in vivo* conditions about 81 % of the selected regenerates exhibited resistance to *Phytophthora parasitica*, whereas none of the control plants showed resistance.

Keywords Culture filtrate (CF) · Resistance · *Citrus jambhiri* and *Phytophthora parasitica*

Abbreviations

2,4-D 2,4-dichlorophenoxy acetic acid
BA 3-benzyl amino purine

NAA Naphthalene acetic acid
ME Malt extract
CF Culture filtrate
PSA Pea sucrose agar medium
MS Murashige and Skoog's medium

Introduction

In Punjab, rough lemon (*Citrus jambhiri* Lush.) is being used as major root stock for a number of *Citrus* spp. like mosumbies, kinnows, oranges, grape fruits etc. for its high vigour and well adaptation to climate of Punjab as well as resistance to *Citrus tristeza virus* (cause of an epidemic). However, the main drawback is its sensitivity to *Phytophthora*, which leads to major losses in an orchard if proper phytosanitary conditions are not followed. *Phytophthora* species have been shown to cause some serious soil borne diseases of citrus including damping off of seedlings in the seedbed, root and crown rot in nurseries, foot rot and brown rot of fruits. This necessitates the production of *Phytophthora* tolerant nursery stock of *Citrus jambhiri* for getting healthier citrus trees with large quantity and good quality of fruits. The conventional breeding approaches to solve such problems of citrus industry have their own limitations like perennial nature of the crop, long juvenility, nucellar polyembryony, heterozygosity etc. Under such circumstances tissue culture techniques offer best possible alternative for improvement and inducing variations and selection of variants for different needs. During the last 3 decades, lot of work has been done on the use of tissue culture methods for selecting the disease resistant plants against different pathogens. These studies have used cell free culture filtrates (CF) or pure toxins of the pathogen or

Savita · G. S. Virk · SavitaA. Nagpal (✉)
Department of Botanical and Environmental Sciences,
Guru Nanak Dev University,
Amritsar 143005, India
e-mail: avinash_nagpal01@yahoo.com

even direct infection by the pathogen for the selection of disease resistance in plants (Hammond-Kosack and Jones 1997 and EL-Kazzaz and Ashour 2004). The present investigation was planned to develop protocol for raising *Phytophthora* tolerant plants of *Citrus jambhiri* through *in vitro* selection system.

Materials and methods

Plant system

Citrus jambhiri plants infected with *Phytophthora* growing in the Botanic Garden of Guru Nanak Dev University, Amritsar (India) were used in the present study. Seeds from the fruits of this plant were collected in the month of September, 2008 for callus induction.

Callus induction

Seeds from the fruits of diseased plant of rough lemon were collected and soaked in water overnight, washed with Teepol for 5 min followed by running tap water for 30 min. These seeds were surface sterilized with 0.1 % mercuric chloride for 15 min and rinsed with autoclaved double distilled water 3–4 times in laminar flow hood. After peeling off both the seed coats, seeds were again sterilized by washing with 0.1 % mercuric chloride solution for 5 min and rinsed with distilled water. Cotyledons were separated from seeds and were injured to induce callus. Injured cotyledons were inoculated on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (2 mg/L) + malt extract (ME) (500 mg/L) and incubated in culture room at 25 ± 2 °C with 16 h photoperiod and 8 h darkness. Calli were multiplied on MS medium supplemented with 2,4-D (2 mg/L) + 3-benzyl aminopurine (BA) (0.75 mg/L).

Pathogen system

Procurement of pathogen culture

Pure culture of *Phytophthora parasitica*, maintained by Prof. T.S. Thind, Department of Plant Pathology, Punjab Agricultural University, Ludhiana (India), was procured for the preparation of culture filtrate.

Multiplication of pathogen culture

To multiply the pathogen culture, Pea sucrose agar (PSA) medium was prepared, containing extract of Pea (125 g), sucrose (10 g) and agar (15 g). Agar plugs (0.5 sq cm) were subcultured on the fresh PSA medium and incubated at 25 °C to get pure culture of *Phytophthora parasitica*.

Preparation of culture filtrate

5 agar plugs (1 sq cm) each of mycelial culture of *P. parasitica* were inoculated in 250 ml of liquid Pea sucrose medium containing salts of MS medium in order to get culture filtrate and placed on shaker for 20 days for continuous agitation at 25–28 °C. After 20 days of initial culture, the pathogen cultures were filtered through Whatman filter paper no.1. pH was adjusted at 5.6 ± 0.2 with 1 N HCL and/or 1 N NaOH. This filtrate was then filtered through Whatman filter paper no. 42 and finally through sintered glass filter (G-4 grade, 0.25 µm pore size) to produce culture filtrate (CF). The pure culture filtrate was transparent in appearance. This culture filtrate was collected and preserved at 4 °C in refrigerator for further use.

Preparation of selection medium

Various concentrations (5–100 %) of culture filtrate (CF) were poured into callus multiplication medium [MS + 2,4-D (2 mg/L) + BA (0.75 mg/L)]. Equal amounts of water from MS callus multiplication medium were replaced by the same amount of CF to prepare concentration of 5–100 % of the selective agent (CF). Three kinds of controls were prepared as follows: two controls of MS medium supplemented with 5 % and 50 % of PS (Pea sucrose) medium instead of equal amounts of water from the MS medium and the third control was MSO as MS callus multiplication medium without culture filtrate.

Bioassay for testing the effect of culture filtrate (Pathogenicity test)

Effect of CF on rooting response was studied by culturing shoots regenerated from calli on MS medium containing 0.5 mg/L naphthalene acetic acid (NAA) and different concentrations of culture filtrate (5–75 %). Two types of controls were conducted, one was rooting medium containing autoclaved culture filtrate and the other was without culture filtrate.

Selection of tolerant calli

The selection protocol was performed with the callus cultures on MS medium containing various concentrations of the culture filtrate of *Phytophthora parasitica*. Such a protocol was targeted in two directions as shown in Fig. 1 (EL-Kazzaz and Ashour 2004). The calli obtained were cultured on selective MS medium supplemented with 5–100 % of CF, to estimate the critical concentration of the selective agent. The survived calli under stress were subcultured for mass propagation for 20 days on callus multiplication medium (2,4-D 2 mg/L + BA 0.75 mg/L) without culture filtrate. After multiplication, these calli were further exposed to second cycle of selection, which

Fig. 1 Steps of selection protocol from lower to higher concentration of CF

contained the same and the 3 steps higher concentrations of the selective agent (CF) and this procedure was repeated several times until the selection regime completed.

Regeneration of selected tolerant calli

The calli selected on each concentration of CF were regenerated on regeneration medium (MS + BA 3 mg/L) containing same concentration of CF on which calli was selected as tolerant.

Rooting of regenerated shoots

For rooting, the regenerated shoots were separated out and cultured on half strength MS medium supplemented with NAA at 0.5 mg/L. In our previous study, NAA at 0.5 mg/L

was shown to give best rooting response of regenerated shoots. For each treatment, 24 culture tubes were inoculated for individual treatment and the experiment was repeated 3 times.

Hardening and acclimatization

Regenerated plantlets were washed with water in order to remove any adhering medium and transferred to autoclaved plastic pots containing a mixture of garden soil, sand and vermiculite in the ratio of 3:1:1. Hardening of potted plantlets was accomplished in culture room set at $26 \pm 2^\circ\text{C}$, 16 h-day-length ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$) by covering them with polyethylene bags to maintain high humidity. After 12–15 days, polyethylene bags were removed initially for a short duration (15–30 min) daily for about one week. Gradually, the daily exposure time was increased by 30 min

Fig. 2 Bioassay for testing the effect of culture filtrate on rooting response of regenerated shoots

Table 1 Bioassay (Pathogenesis test) for testing the effect of culture filtrate on rooting response of regenerated shoots

	Concentration of CF (%)	Per cent survival of shoots Mean±SE ^a	Specific response
^a Out of 24 cultures inoculated for each concentration and each experiment repeated 3 times MS0- MS medium supplemented with 0.5 mg/L NAA without culture filtrate MSA- MS medium supplemented with 0.5 mg/L NAA with autoclaved culture filtrate	MS0	91.66±2.77	Healthy shoots showing rooting
	MSA	86.11±2.77	Healthy shoots showing rooting
	5 %	47.22±2.78	Healthy shoots showing rooting
	10 %	19.45±2.77	Slight leaf fall, showing rooting
	15 %	11.11±2.78	Complete leaf fall and no rooting
	20 %	–	Slight necrosis
	25 %	–	Complete necrosis
	50 %	–	Complete necrosis
	75 %	–	Complete necrosis
	100 %	–	Complete necrosis

for each day. Polyethylene bags were completely removed after 20 days. Subsequently, the plantlets were transferred to earthen pots containing only garden soil and kept in the polyhouse for one month for acclimatization, and then transferred to screen house (Savita et al. 2010).

In vivo testing of the regenerated plants against the pathogen

The selected plants that had regenerated from the resistant callus and unselected tissue culture propagated control plants were subjected to infection under *in vivo* conditions by adding

10 ml of freshly prepared mycelial suspension around each plant. The inoculated plants were observed daily for 15 days following inoculation, and the disease severity was recorded on a A–E scale with A indicating no symptoms (resistant), B indicating slight symptoms (one wilted leaf at the stem base or a brown discoloration of the stem base surface; resistant), C indicating a well-developed, characteristic unilateral wilt of otherwise still healthy plants (moderately resistant), D indicating severe wilt (susceptible) and E indicating a complete wilt death (highly susceptible). 50 plants from control (non-selected) and 50 plants from selected plants were treated with mycelial culture of *Phytophthora*. The experiments were

Table 2 *In vitro* step by step selection of tolerant calli (Somaclonal lines) on different concentrations of culture filtrate of *P. parasitica*

Treatment	First selection cycle % Tolerant calli ^a	Second selection cycle % Tolerant calli ^a	Third selection cycle %Tolerant calli ^a	Fourth selection cycle % Tolerant calli ^a
Control-I MS0	98.67±1.33	98.33±1.67	100.0±0.00	100.0±0.00
Control-II MS1	96.00±2.30	100.0±0.00	100.0±0.00	100.0±0.00
Control-III MS2	94.67±1.33	98.33±1.67	100.0±0.00	100.0±0.00
5 % CF	36.00±2.31	91.67±1.67		
10 % CF	–	41.67±1.67	98.33±1.67	
15 % CF	–	10.00±2.89	38.33±1.67	93.33±0.00
20 % CF	–	–	8.33±1.67	48.33±1.67
25 % CF	–	–	–	31.66±2.89
50 % CF	–	–	–	21.66±1.67
75 % CF	–	–	–	–

^a Out of 25 cultures inoculated for each concentration and each experiment repeated 3 times.

MS0- MS medium supplemented with 2,4-D (2 mg/l)+BA (0.75 mg/l) without culture filtrate (CF).

MS1- MS medium supplemented with 2,4-D (2 mg/l)+BA (0.75 mg/l) with 5 % of liquid PSA medium.

MS2- MS medium supplemented with 2,4-D (2 mg/l)+BA (0.75 mg/l) with 50 % of liquid PSA medium.

First selection cycle: Selection of tolerant calli (somaclonal variation) on different concentrations of CF.

Second selection cycle: Calli selected from 5 % CF multiplied for 20 days on culture filtrate free medium and treated with same and three steps higher concentrations of CF for second selection cycle.

Third selection cycle: Calli selected from 10 % CF multiplied for 20 days on culture filtrate free medium and treated with same and three steps higher concentrations of CF for third selection cycle.

Fourth selection cycle: Calli selected from 15 % CF multiplied for 20 days on culture filtrate free medium and treated with same and four steps higher concentrations of CF for third selection cycle.

Fig. 3 *In vitro* selection of tolerant calli (somaclonal lines) on different concentrations of culture filtrate of *P. parasitica*

repeated three times with a similar trend of results using a completely randomized design.

Results

Bioassay for testing the effect of culture filtrate on rooting of regenerated shoots

Effect of CF on rooting response was studied by culturing shoots regenerated from calli on MS medium containing 0.5 mg/L NAA and different concentrations of culture filtrate (5–100 %). Two types of controls were set: MSA- MS rooting medium containing autoclaved culture filtrate and MS0- MS rooting medium without culture filtrate. When regenerated shoots were cultured on rooting medium without culture filtrate they showed 91.66 % rooting response whereas culture of shoots on MSA medium showed rooting in 86.11 % cultures. A dramatic fall in rooting response (47.22 %) was observed when shoots were cultured on rooting medium containing 5 % culture filtrate. At 10 % CF only 19.45 % shoots showed rooting (Fig. 2). Whereas no rooting response was observed with 15 % and higher concentrations of CF. 100 % necrosis of shoots was observed with 25 % and higher concentrations of CF (Table 1).

Selection of tolerant calli

Three weeks old cotyledon derived calli obtained on MS medium supplemented with 2,4-D (2 mg/L) + ME (500 mg/L) were used for the selection protocol. The calli were first cultured on selective MS medium supplemented with 5–100 % of CF for the selection of critical dose. As shown in Table 2, critical dose of CF for selection of tolerant calli was 5 % (Fig. 3). The calli which survived at exposure to 5 % CF were then subcultured for 20 days on callus multiplication medium free of culture filtrate. At this stage, the protocol was targeted in two directions as shown in Fig. 1. The calli

obtained under the influence of 5 % CF were exposed to the same and three steps higher concentrations of CF for the second selection cycle which means culturing the calli on 5 % and 3 steps higher concentrations (10 %, 15 % and 20 % CF) of the selective agent. At this stage, calli selected in previous cycle showed 91.67 % tolerance to 5 % CF followed by 41.67 % and 10 % tolerance to 10 % CF and 15 % CF respectively. Calli selected on 10 % CF were multiplied for 20 days on CF free medium and further exposed to the same and three steps higher concentrations of CF for third selection cycle. At this stage calli selected in second selection cycle showed 98.33 % tolerance to 10 % CF followed by 38.33 % and 8.33 % tolerance to 15 % CF

Table 3 Regeneration of tolerant calli on medium containing same concentration of culture filtrate on which they were selected as tolerant to culture filtrate of *P. parasitica*

Treatment	No. of cultures tested	No. of cultures regenerated	Per cent regeneration ^a
Control-I MS0	24	20	83.33
Control-II MS1	24	20	83.33
Control-III MS2	24	19	79.16
5 % CF	24	18	75.00
10 % CF	24	16	66.67
15 % CF	24	11	45.83
20 % CF	24	08	33.33
25 % CF	24	0	–
50 % CF	24	0	–
75 % CF	24	0	–

MS0- MS medium supplemented with BA (3 mg/L) without culture filtrate (CF).

MS1- MS medium supplemented with BA (3 mg/L) with 5 % of liquid PSA medium.

MS2- MS medium supplemented with BA (3 mg/L) with 50 % of liquid PSA medium.

^a Regenerated on MS medium supplemented with BA (3 mg/L) and different concentrations of culture filtrate

Fig. 4 Regeneration of selected or tolerant calli (somaclonal lines) on different concentrations of culture filtrate of *P. parasitica*

and 20 % CF respectively. Calli selected on 15 % CF were multiplied for 20 days on culture filtrate free medium and exposed to same and three steps higher concentrations of CF for fourth selection cycle. At this stage calli showed 93.33 % tolerance to 15 % CF followed by 48.33 %, 31.66 %, 21.66 % tolerance to 20 % CF, 25 % CF and 50 % CF respectively.

Regeneration of selected tolerant calli

The calli selected on each concentration of CF were regenerated on regeneration medium (MS + BA 3 mg/L) containing same concentrations of CF on which calli were selected as tolerant. The data in Table 3 revealed that the regeneration ability of the callus cultures decreased by increasing the concentration of CF of *P. parasitica*. Calli selected on 5 % CF showed maximum (75 %) regeneration followed by 66.67 %, 45.83 % and 33.33 % on medium containing 10 % CF, 15 % CF and 20 % CF respectively. However, calli selected on 25 % CF and 50 % CF completely lost their regeneration capacity (Fig. 4). Regenerated shoots were transferred to rooting medium (MS + NAA 0.5 mg/L) which showed maximum 91.67 % rooting response.

In vivo testing of the regenerated plants against the pathogen

The regenerated shoots developed roots on MS medium containing 0.5 mg/L NAA and were hardened at a controlled temperature (25 ± 2 °C) and relative humidity (90 %). These plants showed a relatively higher resistance against *Phytophthora* than the controls. The pathogen resistance of individual plants was scored on A–E scale depending upon the severity of infection. Most of the selected plants were found to fall in groups A and B, whereas all of the control plants belonged to category D and E (Table 4). About 81 % of the regenerates exhibited resistance to *Phytophthora parasitica*, whereas none of the control plants showed resistance.

Discussion

In the present study, *Phytophthora* resistant cultures of *C. jambhiri* were recovered by exposing cotyledon derived calli to CF obtained from the causal agent *Phytophthora*

Table 4 *In vivo* testing of plants for resistance against mycelial culture of *Phytophthora*

Categories	Control (% plants falling in category)				Selected plants (% plants falling in category)			
	I	II	III	Mean	I	II	III	Mean
A	Nil	Nil	Nil	0	Nil	Nil	Nil	0
B	Nil	Nil	Nil	0	78.0	84.0	82.0	81.33
C	Nil	Nil	Nil	0	22.0	16.0	18.0	18.67
D	86.0	82.0	90.0	86.0	Nil	Nil	Nil	0
E	14.0	18.0	10.0	14.0	Nil	Nil	Nil	0

50 plants from control and 50 plants from selected plants were treated with mycelial culture.

A- No symptoms (resistant),

B- Slight symptoms (one wilted leaf at the stem base or a brown discoloration of the stem base surface; resistant),

C- Well-developed, characteristic unilateral wilt of otherwise still healthy plants (moderately resistant),

D- Severe wilt (susceptible) and

E- Complete wilt death (highly susceptible).

parasitica. No phenotypic variation was reported in the selected regenerates. This established a strong correlation between *in vitro* selection and *in vivo* resistance. Our results are in good agreement with reports of El-Kazzaz and colleagues who obtained resistant calli/plants of cucumber via *in vitro* selection under challenging stress of *Fusarium oxysporum* culture filtrate (EL-Kazzaz and Malepszy 1994; EL-Kazzaz and El-Mougy 2007). Similarly many other workers have also reported *in vitro* selection of other plants resistant to different pathogens (Rodriguez et al. 2007; Quaglia and Zazzerini 2007 and Kumar et al. 2008). Such resistance has been reported to result from activation of plant defence responses (Jayasankar and Litz 1998; Salzman et al. 1998; Jayasankar et al. 2000). The resistance to pathogen observed in the present study may be attributed to the induction of mutations (also called as somaclonal variations) within the callus tissues which could be mosaics of sensitive and resistant cells as reported earlier by Arcioni et al. 1987; Chawla and Wenzel 1987a, 1987b; El-Kazzaz et al. 1999. The selection cycle constituted the growth of callus on medium containing CF followed by growing the selected calli on the medium without CF and again on medium with similar and higher concentrations of CF. This leads to production of calli which are tolerant/resistant to the pathogen (CF) because such a cycle will result in decrease of mosaicism and help only the resistant cells to grow on the selective medium. The use of higher concentrations in the selection cycle eliminates the sensitive cells and allows the growth of only the resistant cells. These results are in conformity with those obtained by El-Kazzaz and Abdel-Kader (1998) and El-Kazzaz and Ashour (2004). According to Hammond-Kosack and Jones (1997) resistance can be achieved by the loss or alteration of the toxin target or their detoxification. This indicates that the selected tissues might get altered and produce toxin suppressors or detoxifying enzymes which either suppress or detoxify the CF. Furthermore, the selection protocol using exchangeable cycle of selection on medium with and without CF is considered to be one of the most effective methods and provides assurance about the resistance stability of the cultures or plants (El-Kazzaz and El-Mougy 2001; El-Kazzaz and Ashour 2004). It is also an economical and biosafe method not requiring large space for work and is faster than the traditional breeding program by about 2–3 fold.

Acknowledgement We are thankful to Council of Scientific and Industrial Research (CSIR) New Delhi, Pusa for providing us the financial assistance to complete the experimental work.

References

- Arcioni S, Pezzotti M, Damiani F (1987) *In vitro* selection of alfalfa plants resistant to *Fusarium oxysporum* f.sp. *medicaginis*. Theor Appl Genet 74:700–705
- Chawla HS, Wenzel G (1987a) *In vitro* selection of barley and wheat for resistance against *Helminthosporium sativum*. Theor Appl Genet 74:841–845
- Chawla HS, Wenzel G (1987b) *In vitro* selection for fusaric acid resistant barley plants. Plant Breeding 99:159–163
- EL-Kazzaz AA, Malepszy S (1994) Selection of resistant *Cucumis sativus* regenerated plants to *Fusarium oxysporum* via tissue culture. Proc. of the 1st Egyptian–Italian Sympos. on Biotechnol., Assiut, Egypt. Pp. 121–130
- EL-Kazzaz AA, Abdel-Kader MM (1998) *In vitro* selection of tomato callus culture and regenerated plants on culture filtrate of *Fusarium oxysporum* f.sp. *lycopersici*. Egypt J Phytopathol 26:37–50
- El-Kazzaz AA, El-Mougy NS (2001) Inheritance of disease resistance in cucumber plants to root-rot caused by *Fusarium solani* using tissue culture techniques. Egypt J Phytopathol 29:57–68
- El-Kazzaz AA, Ashour AMA (2004) Genetically resistant cucumber plants to wilt pathogen via tissue cultures. Egypt J Phytopathol 32:1–10
- El-Kazzaz AA, El-Mougy MS (2007) Watermelon resistance to fusarium wilt via tissue culture technique. Arch Phytopathol Plant Protec 1:1–8
- El-Kazzaz AA, Abdel-Kader MM, Hussein MH, Hussein HA (1999) Molecular markers for somaclonal variants of faba beans resistant to root-rot (*Fusarium solani*). Arab J Biotechnol 2:167–180
- Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol 48:575–607
- Jayasankar S, Litz RE (1998) Characterization of embryogenic mango cultures selected for resistance to *Collectotrichum gloeosporioides* culture filtrate and phytotoxins. Theo appl Genet 96:823–831
- Jayasankar S, Zhijian L, Gray DJ (2000) *In vitro* selection of *Vitis vinifera* ‘chardonnay’ with *Elsinoe ampelina* culture filtrate is accompanied by fungal resistance and enhanced secretion of chitinase. Planta 211:200–208
- Kumar JV, Kumari BDR, Sujata G, Castano E (2008) Production of plants resistant to *Alternaria carthami* via organogenesis and somatic embryogenesis of safflower cv. NARI-6 treated fungal culture filtrates. Plant Cell Tiss Org Cult 93:85–96
- Quaglia M, Zazzerini A (2007) *In vitro* screening for sunflower (*Helianthus annuus* L.) resistant calli to *Diaparthel helianthi* fungal culture filtrate. Europ J Plant Pathol 118:393–400
- Rodriguez NV, Kowalski B, Rodriguez LG, Caraballos IB, Suarez MA, Perez PO (2007) *In vitro* and *ex vitro* selection of potato plantlets for resistance to early blight. J Phytopathol 155:582–586
- Salzman RA, Tikhonova I, Bordelon BP, Hasegawa PM, Bressan RA (1998) Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. Plant Physiol 117:465–472
- Savita, Vijay, Virk GS, Nagpal A (2010) Effect of explant type and different plant growth regulators on callus induction and plantlet regeneration in *Citrus jambhiri* Lush. Env We-An Intern J Sci Tech 5:97–106