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In vitro Selection for Resistance Against Purple Blotch Disease of Onion (*Allium cepa* L.) Caused by *Alternaria porri*

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Abstract: Disease tolerant/resistant onion (*Allium cepa* L. cv ADR and ALR) cell lines were selected against purple blotch disease caused by *Alternaria porri*. For this purpose callus and cell suspension cultures derived from mature embryos were exposed to purified toxic culture filtrate produced by the fungus supplemented with MS culture medium. Two selection methods were used: a continuous method in which four cycles of selection were performed on toxic medium whereas during discontinuous method, a pause was given after the second and third cycle of selection using non-toxic medium. Almost 4700 calli obtained from mature embryo and 8300 cell clumps from cell suspension cultures of two onion cultivars were exposed to media with phytotoxin for selection. The discontinuous method proved to be superior as it allowed the calli to regain their regeneration capability. Continuous exposure with toxic culture filtrate resulted up to 78% mortality. *In vivo* pathogenesity test of regenerated plants from the surviving tolerant/resistant cell lines revealed non-sensitive against pathogen toxin. A total 4 lines of ADR and 2 of ALR were documented resistant/tolerant amongst an array of putative resistant/tolerant lines during S_1 generation.

Key words: Embryogenic calli, suspension culture, toxin culture filtrate

INTRODUCTION

The onion (*Allium cepa* L.) is one of the important crops grown in India amongst vegetables and spices. At present, breeding for resistance to biotic and abiotic stresses is a high priority. Conventional breeding programmes including extensive intermating and screening campaign helps breeder to improve cultivars, however, this is limited by inherent difficulties, open pollination, high level of heterozygosity and poor fertility of F_1 hybrids. Moreover, unavailability of disease/pest resistance genes in gene pool makes breeder task more difficult. *In vitro* selection against disease causing phytotoxin offers an alternative means of screening disease resistant cell lines.

In vitro selection offers an immense potential for the quick and comprehensive generation of useful somaclones or mutants for resistance against various biotic and abiotic factors. These plants may serve as an excellent donor of the resistance gene(s) in breeding programmes. Selection in plant cell cultures using pathotoxins as a screening agent for resistance have been reported in *Brassica napus* to *Phoma lignum* (Sacristan 1985), in potato to *Phytophthora infestans* (Behnke 1979) and *Fusarium oxysporum* (Behnke 1980, Schuchmann 1985), in tobacco to *Pseudomonas syringae* and *Alternaria alternata* (Thanutong *et al*., 1983), in alfalfa to *Fusarium oxysporum* (Hartman *et al*., 1984), in maize to culture filtrate of *Helminthosporium maydis* (Gengenbach and Green 1975) and in groundnut (Venkatachalam *et al*., 1998, Ashok *et al*., 2004).

In practice involvement of *in vitro* selection techniques in the crop improvement programme is very limited. Except for few, most of the reports suggest negative correlation between *in vitro* and *in vivo* responses for resistance. Reason for this lies with several factors such as plant species, genotypes, source of the explant and nutritional and environmental factors. Moreover, expression of resistance or susceptibility may require specific cell types/tissue arrangements or physiological function not present in the cultures.

Although, a few studies have been conducted to obtain prolific *in vitro* selection, no work has been reported so far on onion in the world. During the present investigations an effort has been made for selection against purple blotch disease of onion.

MATERIALS AND METHODS

Plant material: Two purple blotch susceptible onion cultivars namely Agrifound Dark Red (ADR) and Agrifound Light Red (ALR) were selected to carry out the present study. An efficient *in vitro* plant regeneration protocol from callus and cell suspension cultures were

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optimized before conducting the *in vitro* cell line selection experiments using toxic culture filtrate. Callus and suspension cultures were established from mature embryos (Tiwari *et al*., 2004, 2007).

For raising embryogenic callus and suspension culture a series of growth regulators were supplemented to basal MS (Murashige and Skoog, 1962) medium. All culture media combinations were short-listed on the basis of previous work conducted by various scientists and preliminary experiment of this laboratory. The basal MS media supplemented with different concentrations of plant growth regulators in various combinations (NAA, 2,4-D, picloram, kinetin, zeatin, TDZ and BA alone as well as kinetin, zeatin, TDZ and BA in combination with NAA, 2,4-D and picloram). Unless specified, all the media contained MS macro and micronutrients, vitamins, 30.0 g L^{-1} sucrose and 7.5 g L^{-1} agar only for semi-solid medium. All culture medium were autoclaved at 121°C under 1.1 kg cm^{-2} for 20 min after adjusting the pH to 5.6±0.1 with 1N KOH. MS basal medium and all other add-ons were procured from HiMedia® Laboratories, Mumbai, India.

For callus culture, mature seeds were surface sterilized with 70% (v/v) ethanol for 1 min followed by a treatment with 5% (w/v) sodium hypochlorite for 15 min and were finally rinsed 3 times with sterile double distilled water under aseptic conditions. Surface sterilized seeds were soaked for 24 h in sterile double distilled water. Mature embryos from pre-soaked seed were excised and cultured on the explant inoculation medium in 100×17 mm glass petridishes. For raising suspension cultures, six to eight weeks old embryogenic calli obtained from mature embryo culture were transferred in to MS liquid medium. The cultures were agitated on a horizontal shaker at 140 rpm, at 25±2°C, under the complete darkness. Every 15 days the old medium was replaced by an equal volume of the fresh medium.

Isolation and purification of pathogen: *A. porri* isolates from infected plants were collected from different locations. Leaves with infected lesions were surface sterilized with $HgCl_2$ solution and thoroughly washed with sterile double distilled water before inoculation. Inoculated plates with Potato Dextrose Agar (PDA) medium were kept for incubation at 25°C. The fungal colonies appearing on plates were subcultured, purified and maintained at 25°C.

Pathogenicity test: The virulence of the isolate of *A. porri* was tested by seedling leaf injury method. Leaves from 25 cm tall seedlings were gently injured by sterilized needle and mycelial mat was spread on the injured leaves. Inoculated seedlings were incubated in plastic bell jars under high humidity. After 10 to 15 days, virulent strains revealed typical purple to brown blotches were observed on the inoculated leaf surface.

Preparation of 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 every 4 weeks. Ten to fifteen pieces of PDA cut from a two-week-old culture of *Alternaria porri* were inoculated in 250 mL Erlenmeyer flask containing 50 mL liquid MS medium. Two weeks later medium was divided into five equal portions of 10 mL. Cultures were incubated under dim light at 22°C for six weeks. After 5-7 weeks small mycelium balls emerged in a uniform suspension. The suspension was passed through filter paper Number1 (Whatman) and culture filtrate was then subjected to centrifugation at 10,000xg for 20 min. Supernatant was sterilized using nitrocellulose filter (0.22 μm) and stored at -20°C.

Determining the LD_{50} of *A. porri* toxic culture filtrate: To determine the selection concentration of phytotoxin, calluses were separated into small pieces and were placed on varying concentrations of toxin. LD_{50} was established with reference to approximately 50% retarded growth of callus. Fresh weight and relative growth rates of callus and suspension culture on seven different levels of phytotoxin added in initial culture medium were determined after 4 weeks (Fig. 1A-D and Table 1).

***In vitro* selection and regeneration procedures:** For initiation and selection of disease tolerance, small pieces of embryogenic calluses and suspension cultures were subjected to fortified MS culture medium supplemented with varying levels of toxic culture filtrate and 0.5 mg L^{-1} each of NAA, kinetin and BA. After continuous and discontinuous selections, tolerant/resistant calli were grown on MS medium with 7.8 mL^{-1} toxic culture filtrate to select the disease tolerant cell lines. After two weeks of plating, *A. porri* culture filtrate toxin tolerant colonies with considerable growth were selected (Fig. 1 E). Following selection on toxic medium, surviving callus obtained from embryo cultures and embryoids acquired from suspension cultures were grown on MS maintenance medium without growth regulators and parts of survived callus were placed on MS regeneration medium fortified with 0.5 mg L^{-1} each of NAA, Kn, BA and 20 g L^{-1}sucrose (Fig. 1 F-G). Regenerates 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 transferred under 30±2°C and 60±5% RH for 15-20 days in greenhouse for acclimatization.

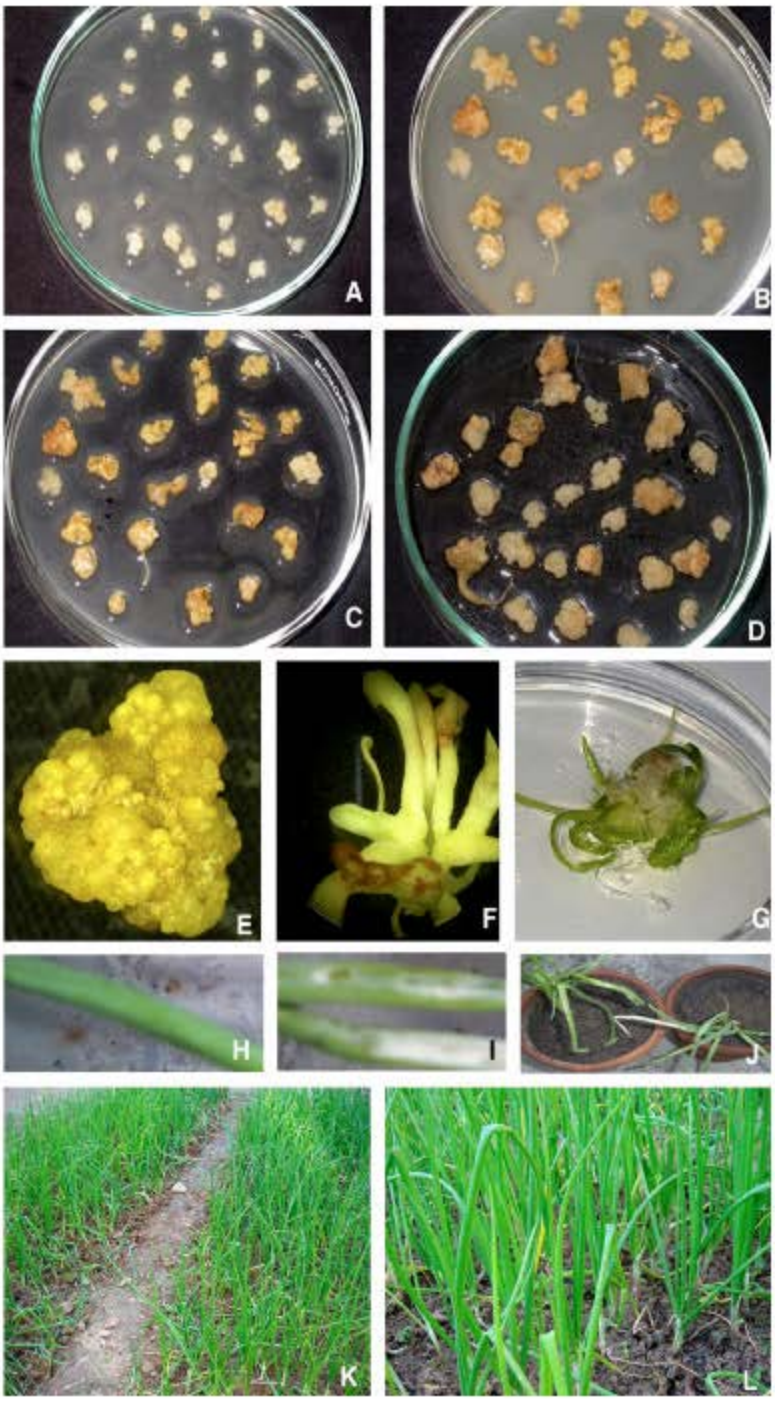

Fig. 1: *In vitro* selection in onion cv ADR and ALR: A-D. Effect of varying concentration of *Alternaria porri* culture filtrate on onion callus cultures; E. Callus proliferated on tolerant/resistant callus; F-G. Plant regeneration from cultures tolerant to *A. porri*; H-J. *In vivo* testing of putative tolerant plants: H. Uninoculated leaf; I. Inoculated leaves J. putative tolerant and susceptible plants against *A. porri*; K-L. Field trial of putative resistant/tolerant plants of cv Agrifound Dark Red and Agrifound Light Red.

***In vivo* testing of regenerated plants against pathogen:** Leaves of regenerated putative disease tolerant and control plants were sprayed with 70% ethanol. After approximately 30 min dried leaves were inoculated with 10 μL spore suspension and kept under 60±5% RH. In susceptible and control plants, the purple blotches appeared on the surface of leaves within 3-4 days of inoculation. The plants regenerated from *in vitro* cultures were categorized as resistant, intermediate and susceptible (Fig. 1 H-J).

Evaluation of onion plants, developed by *in vitro* selection against purple blotch disease: The two onion varieties ADR and ALR either developed by embryo culture or by suspension culture were selected at LD_{50} of *A. pori* toxic filtrate. To observe their performance, plants were placed under field conditions (Fig. 1 K-L). The fungal inoculum was artificially sprayed during the initial vegetative phase. The data on disease incidence was recorded after 30 days by rating the size of the spots in to different grades. The Percent Disease Intensity (PDI) was calculated as under.

$$PDI = \frac{\text{Sum of numerical rating}}{\text{Total No. of leaves} \times \text{Maximum rating value}(4)} \times 100$$

RESULTS AND DISCUSSION

In vitro selection is often hampered by difficulties in regenerating plants in long culture phages from *in vitro* selected calli against toxins and lower expressibility of selected traits in the regenerated plants in culture systems. The present experimental evince that *in vitro* regeneration of rather recalcitrant species like onion can be improved upon and can become amenable to *in vitro* selection.

During embryo and suspension culture, Tiwari *et al.* (2004, 2007) observed significant differences among different growth regulator type concentrations and their interactions with each genotype for callus induction, embryoid formation and plantlet regeneration. For callus culture, MS medium fortified with 2.0 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} BA substantiated superior for all culture phases. In case of suspension cultures, the liquid medium containing 4.0 mg L^{-1} 2, 4-D in combination with 0.5 mg L^{-1} BA was found the most competent. For subsequent subculturing reduced concentration of 2,4-D (2.0 mg L^{-1} 2, 4-D in combination with 0.5 mg L^{-1} BA) was supported faster development of embryos. Frequent plantlets were regenerated on solid MS medium supplemented with 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BA, 0.5 mg L^{-1} kinetin, 20 g L^{-1} sucrose and 7.5g L^{-1} agar. Cultivar ADR was found more responsive than ALR for the both culture systems.

During the present investigation, the culture filtrate of the pathogenic fungus was used as the source of crude toxin preparations. At higher toxin concentration (8.1 mL L^{-1}), almost mortality was observed. At minimum level of toxic culture filtrate (below to 7.5 mL L^{-1}), significant effect on callus growth was not observed (Fig. 1A-D and Table 1). Thus, LD_{50} (7.8 mL L^{-1}) of toxin concentration was used for selection criteria. The control callus and the one grown at the lowest concentration of fungal toxin looked healthy and survived considerably, whereas, the calli treated with the highest toxin level showed a brown colour and eventually 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.

Toxin-induced leakage of electrolytes from tissues suggests plasmalemma as the site of action and toxin induced electrolyte leakage from leaves (Rudolf, 1976; Yoder, 1983). During the present investigation, with the dilution of the toxin, there was a gradual decrease in electrolyte leakage from calli and leaves. The identical response of calli and leaf tissues to toxic culture filtrate indicates that physiological and molecular testing and selection for disease resistance is feasible at callus phase (Fig. 1E).

During continuous method, after the first cycle of selection of callus cultures on the toxic medium, onion cultivars ADR and ALR showed 37.2 and 33.3 % survival rate respectively (Table 2). However, during the second cycle about 5% 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 27.7% callus survival for cv ADR and 23.6% for cultivar ALR after 4 cycles of selections. During discontinuous method, 29.3% calli of cv: ADR and 26.4 % calli of cv: ALR survived.

With the continuous method applied with cell suspension cultures, like callus culture selections resulted in 34.61% surviving embryoid of ADR and 31.26% of ALR after the first cycle of selection on the medium with toxic culture filtrate (Table 3). Similarly, 4-5% of the calli still died during the second cycle before acquiring insensitivity during the third cycle. Thereafter only few embryoids (2-3%) died due to toxic culture filtrate present in medium during the fourth round of selection. Total of embryoid 25.38% from ADR and 22.09% from ALR survived after 4 cycles of selection by continuous method. Whereas, selection by discontinuous method finally survived 27.26% and 24.28% embryoids of cv ADR and ALR, respectively.

Table 1: Comparison of growth rates 1 of mature embryo derived calli from callus and cell suspension of onion on different levels of toxin concentration in growing medium

	Relative growth rate of callus cultures 2		Relative growth rate of suspension cultures 3	
Phytotoxin (mL L^{-1})	ADR	ALR	ADR	ALR
0.00	100.00±0.40	100.00±0.40	100.00±0.40	100.00±0.40
7.5	87.34±0.40	84.91±0.40	88.52±0.40	80.78±0.40
7.6	67.48±0.28	65.13±0.31	74.86±0.32	67.86±0.36
7.7	62.52±0.28	64.68±0.31	66.34±0.32	61.52±0.36
7.8	30.12±0.36	32.19±0.40	32.40±0.40	29.18±0.44
7.9	13.78±0.32	11.98±0.30	15.84±0.30	12.56±0.32
8.0	9.54±0.32	8.75±0.30	10.62±0.30	9.90±0.32
8.1	2.77±0.23	0.50±0.13	1.08±0.11	3.67±0.19

Mean was obtained from weights of five inoculum/ treatments after 4 weeks, Callus and cell suspension cultures were cultured on MS medium fortified with 2.0 mg L^{-1} 2, 4-D, 0.5 mg L^{-1}BA and different levels of toxic culture filtrate, Callus and cell growth appeared average fresh weight (mg) of 5 calli

Table 2: Response of mature embryo derived calli of onion to the toxin preparation of *Alternaria porri*

		No. of surviving calli after 4 selection cycles			
Cultivar	No. of calli	I	II	III	IV
Continuous method					
ADR	1000	372 (37. 2%)	326 (32.6%)	297 (29.7%)	277 (27.7%)
ALR	1100	363 (33.3%)	313 (28.4%)	278 (25. 2%)	260 (23.6%)
Discontinuous method					
ADR	1200	469 (39.1%)	-	-	352 (29.3%)
ALR	1400	534 (38.1%)	-	-	369 (26.4%)

Table 3: Response of clumps obtained from embryogenic suspension culture of onion to the toxin preparation of *Alternaria porri*

		No. of surviving calli after 4 selection cycles			
Cultivar	No.of calli	I	II	III	IV
Continuous method					
ADR	1875	649 (34. 61%)	577 (30.76 %)	513 (27.36%)	476 (25.38%)
ALR	2050	641 (31.26%)	539 (26.29%)	476 (23. 21%)	453 (22.09%)
Discontinuous method					
ADR	1995	726 (36.39%)	- (27.26%)	-	544
ALR	2446	859 (35.11%)	- -	-	594 (24.28%)

Similar to experiment at issue, Chawla and Wenzel (1987) performed *in vitro* selection of wheat and barley by applying intense initial selection pressure of toxin to kill about 70% of the calli and subsequently increased the concentration of toxin in the media. Similarly during the present investigation, first cycle selection resulted in 65-70% callus mortality in both the cultivars. A discontinuous method of selection was experimented in onion to prolong regeneration capability of cultures on toxic medium. Results after completing four cycles of selection by continuous and discontinuous methods were almost similar however, discontinuous method exhibited apparent advantage of high regeneration ability over the continuous method.

In vivo testing of 51 regenerated plants of ADR to the pathogen have revealed 4 resistant plants, 29 intermediately tolerant plants with very few brown spots and 18 susceptible plants. Likewise, 32 regenerated plants of ALR have revealed 102 plants resistant to the pathogen, 21 intermediate and 9 susceptible (Fig. 1 H-J and Table 4). Exposure and selection of callus cultures to toxin resulted in plants that were tolerant to the pathogen. Variation in reaction to the pathogen observed during present experiment indicates that the tolerance to *Alterneria porri* in onion is probably due to changes in nuclear genome however, confirmation at molecular level and subsequent generation is required.

The response of regenerated plants was recorded as the size of the spot on the leaves the plants were then categorized into different grades after calculating the PDI as described earlier. Based upon the PDI value, the plants were categorized and counted, as resistant (<10), moderately resistant (11-25), moderately susceptible (26-40) and susceptible (>40). Thirty days after transplanting

Table 4: Regeneration frequency of onion calli tolerant/resistant to phytotoxin and *in vivo* reaction of regenerated plants to *Alternaria porri* phytotoxin

			In vivo response of regenerated plants to disease†			
Cultivars	Calli/clumps	Plants regenerated (albino)	Plants tested	R	I	S
ADR	990	134 (15)	51	04	29	18
ALR	1000	113 (22)	32	02	21	09

†R: Resistant; I: Intermediate; S: susceptible

Table 5: Performance of the onion plants against purple blotch disease under field conditions

	var. Agrifound Light Red (ALR)		var. Agrifound Dark Red (ADR)	
Percent disease intensity (PDI)	No. of plants raised by embryo culture	No. of plants raised by suspension culture	No. of plants raised by embryo culture	No. of plants raised by suspension culture
1-10 (Tolerant)	01	03	00	02
11-25 (Moderately tolerant)	08	03	09	02
26-40 (Moderately susceptible)	13	05	06	04
>40 (Susceptible)	18	14	23	09

transplanting, final stand of the plants was counted 402 and 201 for ALR during embryo and suspension culture respectively and 318 and 180 for ADR. Data on PDI is presented in the Table 5 and Fig. 1 K-L indicates that embryo culture of cv ALR on culture medium with toxic culture filtrate regenerated only one resistant plant and 03 plants raised from suspension culture confirmed resistance, whereas remaining plants have shown varying degree of reaction against purple blotch disease. Similarly, from cv ADR, only 02 plants raised by suspension culture exhibited tolerance whereas, none of the plants raised from embryos revealed tolerance against purple blotch disease. Such plants will be grown in the next season to confirm tolerance. The seeds of these single plants have been collected so as to confirm performance under natural conditions.

This approach of generating disease tolerant/resistance raises question weather the trait acquired is due to the mutation or somaclonal variation. Many plant *species* earlier have been selected for disease tolerant/resistance using culture filtrate or partially purified toxins (Song *et al*., 1994; Gangaotti *et al*., 1985, Sunlihua and Lu, 1997). Selection of disease resistant plants without other apparent mutations has been accomplished in rice by short-term exposure of calli to *Helmenthosporium oryzae* toxin followed by regeneration on toxin free medium (Vidyasekaran *et al*., 1990). It can be concluded from the present study that the sensitivity of the cultured cells to the toxic culture filtrate of *A. porri* is related to the susceptibility of the onion regenerants to the pathogen and the selection protocol using the toxic culture filtrate of *A. porri* produced purple blotch disease resistant plants. Similar results in groundnut for *C. personatum* (Venkatachalam *et al*., 1998) and tobacco for *A. alternate* (Thanutong *et al*., 1983) have proved useful in identifying resistant plants for corresponding pathogens using culture filtrate strategy. Hence, it could be a method of choice for developing fungal disease resistant genotypes in onion and other crops.

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