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Biochemical Characterization of Rice Somaclones Resistant to Blast

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Abstract: The aim of the present study was to produce rice somaclones resistant to *P. oryza* that could be useful to improve rice plants against blast disease and use them in the future breeding programs. In addition, to detect biochemical changes among somaclones and their original cultivars. Mature embryo-derived calli of the three susceptible rice varieties, Sakha 101, Sakha 104 and Riho were stressed with different concentrations of fungal toxin filtrate. Blast-resistant lines of rice were developed by *in vitro* selection with toxin filtrate and afterward, their callus induction and their regeneration were tested. The percentage of regenerating calli greatly varied depending on variety and toxin concentration. The reduction of regeneration percentages induced by toxin filtrate was more pronounced in the more sensitive variety, Riho. Protein electrophoresis of regenerated plants which were tested for blast resistance in the R2 generation in the greenhouse, revealed six additional protein bands as compared to their original susceptible cultivars. Esterase patterns showed new enzyme bands in the resistant R2 plants, Alcohol dehydrogenase patterns displayed two distinct bands at two activity zones; these patterns possibly occurred due to a genetic activation mechanism which affected blast gene(s) expression. The blast resistant lines should be tested under field conditions before using them in rice blast breeding programs.

Key words: Fungal toxin filtrate, *in vitro* selection, *Oryza sativa*, *Pyricularia oryza*, somaclonal variation

INTRODUCTION

Rice blast caused by *Pyricularia oryza* is one of the major yield constraints in the world and Egypt as well. Breeding for blast resistance is the most economical mean to reduce grain yield losses. *In vitro* selection of plant cells and tissue has attracted considerable interest over the recent years because it provides the means to study the biochemical and genetic processes of plants. Regenerated plants are expected to have the same genotype as the donor plant, however, in some cases somaclonal variants have been found among the regenerated plants (Karp *et al*., 1987). Mature as well as immature embryos are currently the most reliable and efficient target tissues for *in vitro* regeneration of cereals (Chang *et al*., 2003).

Somaclonal variation results in the production of new genotypes with a limited change in the original genome. Somaclonal variation is apparently caused by gene amplification, the alteration of a basic couple, transposing migration, methylation transform, chromosome instability, chromosome inversion, one spot mutation, translocation, ploidy change, restructuring or deletion (George and Sherington, 1984; Phillips *et al*., 1990; Dennis, 2004). Its effectiveness and efficiency are due to its ability of changing the plant to the desired character, either by applying a selection agent on the culture media or by giving particular condition to change the somaclone with the required character (Van den Bulk, 1991; Karp, 1995).

In vitro screening was described for first time in regenerated tobacco plants resistant to *Psedomonas tabaci* by Carlson (1973). Similar work hase been reported with arrange of diseases including *Helminthosporium oryzea* in rice (Vidhyasekaran *et al*., 1990). It is important to device an efficient protocol of callus proliferation in order to start *in vitro* selection for biotic stress, such as fungal toxin filtrate, and to broaden the opportunities for genetic manipulation of rice through tissue culture. The aim of the present study was to produce rice somaclones resistant to *P. oryza* that could be useful to improve rice plants against blast disease and use them in the future breeding programs. In addition, to detect biochemical changes among somaclones and their original cultivars.

MATERIALS AND METHODS

Callus induction and plant regeneration: This study was conducted at the Department of Genetics, Faculty of Agriculture, Kafrelsheikh University during the years 2010 and 2011. Three susceptible rice cultivars, namely Sakha 101, Sakha 104 and Riho were used in this study. Mature dehusked seeds were surface sterilized with 70% ethanol for 1 min followed by 20 min. treatment in 50% sodium hypochlorite. Treated seeds were rinsed twice with sterile distilled water and blot dried onto a filter paper. For callus induction, sterilized seeds were plated on MS (Murashige and skoog, 1962) medium supplemented with 2.0 mg/L 2, 4-D, 0.5 mg/L Kin, and

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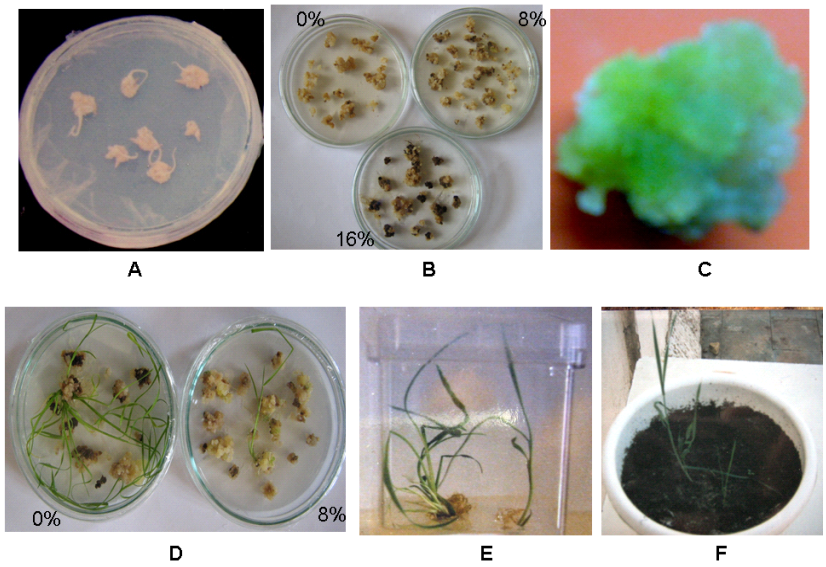

Fig 1: Different stages of callus induction and plant regeneration from mature embryo in Rice, A) Callus induction, B) Callus proliferation under different toxin filtrate concentration, C) Nodular structure of the callus on regeneration medium, D and E) Regenerated plants, F) acclimatized plants

30 g/L sucrose with different concentration of fungal toxin filtrate (0, 8, 16 and 24%). The media were solidified with 0.7% agar. The pH of all media was adjusted to 5.8 before autoclaving at 121ºC for 15 min. The media (20 mL) were poured into Petri dishes and 10 seeds were cultured per Petri dishes in three replications. The culture incubated at 25±2ºC in darkness for three weeks. Only induced calli were divided into small pieces and subcultured into callus induction medium with different concentration of toxin filtrate, three weeks each. Survival calli were cultured in selective regeneration MS medium supplemented with 2 mg/L Kin and 0.5 mg/L IAA and different concentration of toxin filtrate. The cultures were kept at 25ºC under 16/8 light/dark photoperiodic regime for 2 moths with 2 interval subcultures.

Regenerated plantlets (R1) were acclimatized and transplanted to pots. These plants were grown to maturity under greenhouse conditions. Seeds were harvested from individual plants.

Greenhouse experiments: Greenhouse experiments were conducted in plastic trays (30 × 10 × 15 cm) The genotypes of the R2 population evaluated in this study included three sensitive parents (Sakha 101, Sakha 104 and Riho). Seeds harvested from R1 plants of each genotype were sown in 10 rows per tray. Twenty one days old R2 plants, were inoculated with the aqueous suspension of spores of the *P. oryza* isolate No.120 at the concentration of 3 × 105 spores/ml. The plants after inoculation were incubated in the moist chamber for 24 h before transferring them to greenhouse benches at temperature varying from 25-29ºC. Leaf blast evaluations were made seven to nine days after inoculation using a visual rating scale (0-9) according to Leung *et al*. (1988). Seedlings showing susceptible reaction were removed from the trays and the remaining resistant seedlings (0-1) were used for biochemical analysis.

Biochemical analysis: Protein extracts were prepared from 50% expanded young leaves Homogenates were obtained by mechanically grinding with sucrose 20%. Protein extracts were centrifuged at 14,000 rpm for 10 min at 4ºC and apply in 12% polyacrylamide gels according to Laemmli (1970), and stained by commase blue dye. Enzyme activity of alcohol dehydrogenase (ADH) and esterase (EST) were visualized by using the staining procedures of Soltis *et al.* (1983) for EST and according to (Schwartz and Endo, 1966) for ADH. The Rf (Relative electrophoretic mobility) was calculated for each isozymic band.

RESULTS

Callus induction: Mature embryos explants exhibited an initial swelling followed by callus formation within three weeks of incubation (Fig. 1). It was noted that callus proliferation started on MS basal medium with different concentration of toxin filtrate (0, 8, 16 and 24%). Callus induction frequency and calls fresh weight decreased as the concentration of fungal toxin filtrates increased for the three tested rice cultivars. The callus induction frequencies was 100% under control conditions (0% toxin filtrate), but it varied between the three cultivars under toxin filtrate. Sakha 101 gave the highest value for callus

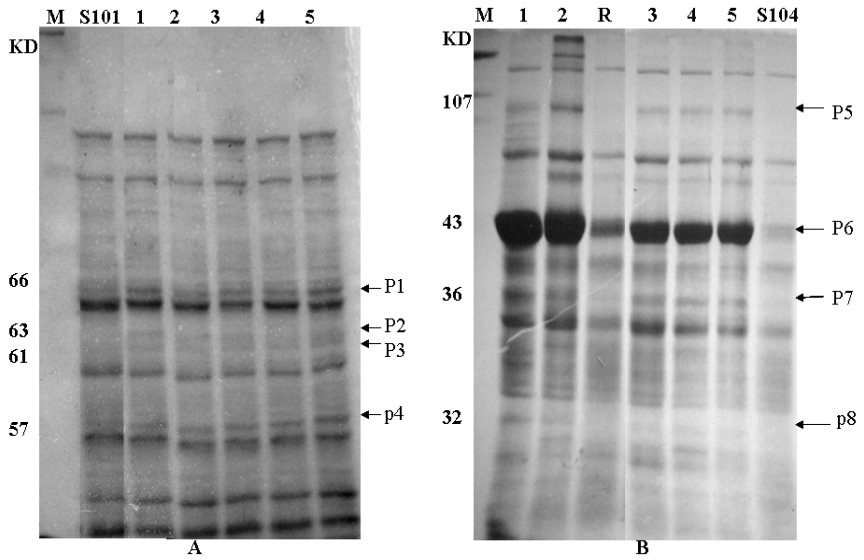

Fig. 2: Electrophoresis protein pattern of Sakha 101 (S101) and its regenerants (A), Riho (R) and Sakha 104 (S104) and their regenerants 1, 2, 3, 4 and 5, respectively

Table 1: Mean values of callus induction (%) and callus fresh weight (g) under different concentration of toxin filtrate.

Genotypes	Toxin filtrate (%)	Callus induction (%)	Callus fresh weight (g)
Sakha 101	0	100.00	1.53
	8	99.81	0.93
	16	96.00	0.35
	24	37.00	0.13
Sakha 104	0	100.00	1.35
	8	99.00	0.51
	16	65.33	0.22
	24	25.50	0.08
Riho	0	100.00	1.31
	8	57.33	0.37
	16	25.50	0.02
	24	0.33	0.01

induction and callus fresh weight followed by Sakha 104 and the lowest callus induction recorded by the most susceptible cultivar Riho under 8%, 16% and 24% toxin filtrate respectively (Table 1).

Plant regeneration: Stressed calli induced at 8% toxin filtrate were placed onto regeneration media containing various concentrations of toxin filtrate. The embryogenic calli that present nodular structures (Fig. 2) developed into shoot buds after 4 weeks. The regenerated calli were subcultured in the same medium to produce entire plants (Fig. 2). The percentage of regenerating calli greatly varied depending on variety and concentration of toxin filtrate. The extent of reduction of regeneration percentages induced by high toxin concentration as a function of variety correlated well with the reduction of plant survival, no plants were produced under higher concentration of toxin filtrate (16 and 24%) since the majority of callus died under these condition (Fig. 1).

The result revealed that highest percentage of plant regeneration of Sakha 101 followed by Sakha 104 and the lowest for Riho. Furthermore, regeneration ability decreased as toxin filtrate increased. Toxin filtrate concentration of 8% was the best to test blast resistance while 16 and 24% prevented plant regeneration (Table 2).

Biochemical analysis: The genetic differences among the cultivars and its regenerates (somaclones) under different concentration of toxin filtrates were illustrated using biochemical markers (isozyme and protein). Four new protein bands (P1, P2, P3 and P4) were induced in Sakha 101 regenerants while absent in original cultivar (Fig. 2) In the same manner, protein pattern of Riho (R) and Sakha 104 (S104) and their regenerates revealed that protein bands (P5 and P8) were found in all regenerates but absent in original cultivars. Moreover, the activity of bands P6 and P7 were higher than their corresponding original cultivars. (Fig. 2).

In the present study, esterase pattern was done to determine the biochemical variation in rice somacolnes. From 2 to 4 new esterase enzyme bands were induced in the resistant R2 seedlings compared to their corresponding susceptible parents, furthermore the number and activity of the induced bands varied among the resistant regenerants (Fig. 3). Somaclones regenerated from Sakha 104(S104) cultivar represent two newly induced isozyme bands (E3 and E5), followed by Riho regenerants that contained three new bands (E4, E6 and E7), whereas the highest number of induced bands, 4 (E3,

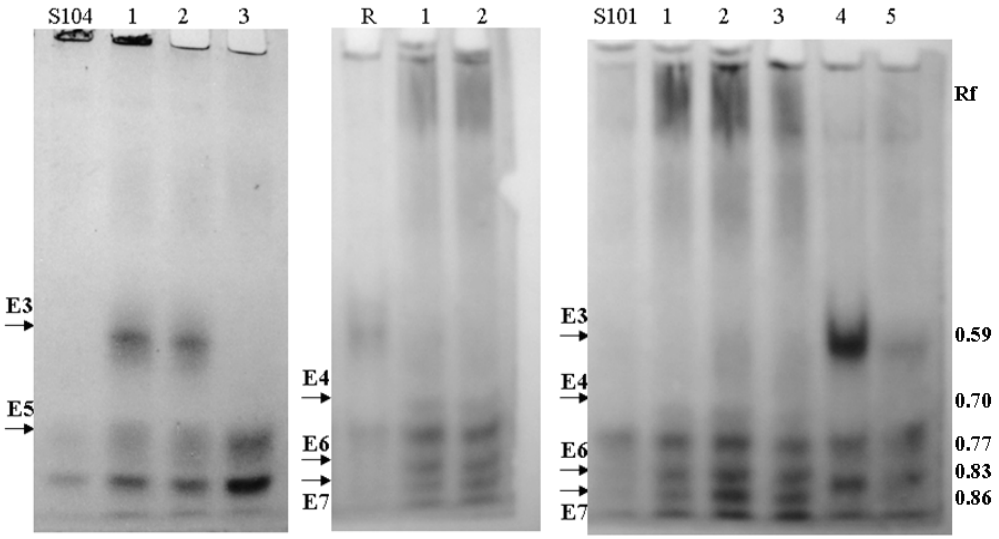

Fig. 3: Esterase banding pattern for the three susceptible rice cultivars and their regenrants (S104) Sakha 104, (R) Riho and (S101) Sakha 101. Rf, (Relative electrophoretic mobility)

Table 2: Embryo culture response characters under different concentration of toxin filtrate.

			Responded calli for plant regeneration		Total plant production	
		Plated calli				
Genotypes	Toxin filtrate (%)	No	No	%	No	%
Sakha 101	0	90	23	55.55	45	50
	8	80	12	15	9	11.25
	16	75	1	1.33	0	0.00
	24	85	0	0.00	0	0.00
Sakha 104	0	101	17	16.83	19	18.81
	8	95	9	10.34	5	6.89
	16	105	0	0.00	0.	0.00
	24	86	0	0.00	0	0.00
Riho	0	65	8	14.28	10	15.38
	8	56	6	9.47	4	5.26
	16	52	0	0.00	0	0.00
	24	45	0	0.00	0	0.00

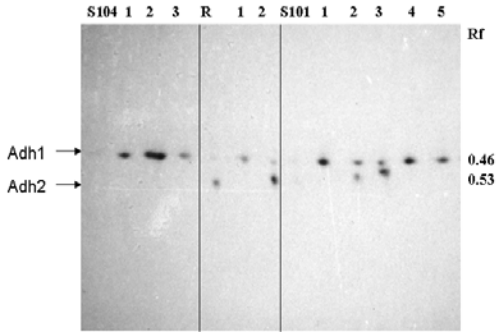

Fig. 4: Alcohol dehydrogenise banding pattern susceptible rice cultivars and their regenrants (S104) Sakha 104, (R) Riho and (S101) Sakha 101. Rf, (Relative electrophoretic mobility)

E4, E6 and E7) were recorded for the cultivar Sakha 101 (S101) regenerants.

Gels stained for Alcohol dehydrogenase ADH activity displayed two distinct bands at two activity zones (Fig. 4). Band 'A' type occurred most frequently (70.8%) followed by 'B' type (30%). The bands could be corresponded to *Adh* with two alleles according to the migration on gel. Type 'B' allele with least frequency could be a rare allele. Type 'A' allele was common in all tested regenerants except original cultivars.

DISCUSSION

Callus induction and plant regeneration efficiency in the three susceptible rice varieties were relatively low. These results are in agreements with Xie *et al*. (1990) reporting that some rice cultivars such as Tetep and Taipei 309 were tissue culture stable and seldom produce variants. According to Evans and Gamborg (1982), callus was cultured only for 45 days in induction medium to obtain a limited change for one trait such as disease

resistance because much of the variation is proportional to the duration of culture induction. 2, 4-D was used in rice callus induction as mentioned by Yoshida and Kato (1996), found that multiplication media supplemented with 2, 4-D produced calli and shoot primordial.

Although attempts to select variants in tissue culture for resistance to rice blast are very few and the results are conflicting (Pachón, 1989; Xie *et al.*, 1990), many promising rice germplasm with resistance to disease have been discovered from the mutants of susceptible rice varieties through *in vitro* somaclonal mutant selection (Ling *et al*., 1986; Mandal *et al*., 1995; Hemalatha *et al*., 1999). This has proved the utility and efficiency of this technique in exploring novel rice germplasm resources for disease resistance.

Many researchers have explained that tissue culture induced variation or genetic changes based on the changes on DNA level which ultimately is reflected in differences in protein or enzyme forms. Moreover, Galussi *et al.* (1996) found two different electrophoretic protein models in another rice variety. Furthermore, the polymorphisms of ADH and EST patterns possibly occurred because the genetic repression mechanism that controlled gene expression was affected (Frías de Fernandez *et al.*, 1975), or because of changes due to post translational modifications (Acquaah, 1992). ADH and EST patterns of regenerated rice plants showed polymorphisms compared with plants of the original varieties (Medina *et al*., 2004).In the same manner, Waspi *et al*. (1998) found that the accumulation of *Pir7b* transcripts, which correspond to one of the defense-related genes that encodes an esterase in cultured rice cells. Furthermore, Davies *et al.* (1986) reported that out of 600 regenerants of *Triticum aestivum*, 13 plants revealed isozyme variation in ADH patterns.

The biotic stress and chemical environment of the cultured cell causes stress on the cell, so different genome interact with such stress signals differently and produce response of different magnitude to the culture cell and may undergo many changes in the gene level. Those changes express differently in biochemical characters of plants obtained through *in vitro* selection under biotic stress such as toxin filtrate of *P. oryza*. Another explanation could be Alterations in protein patterns have been associated with signal transduction events leading to defense responses in cultured plant cells as well as in whole plants (Farmer *et al*., 1991; Felix *et al*., 1991; Raz and Fluhr, 1993).

No visible morphological variation was found in the somaclones that showed differences in protein and isozyme patterns with respect to their original cultivars, as reported by Noh and Minocha (1990). The obtained rice somaclones were able to perceive a multitude of rice blast toxin filtrate and respond to them in various ways, including the activation of defense-related genes.

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