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Antioxidant and *In situ* Chitinase gel activity in Safflower *(Carthamus tinctorius* L)

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Abstract: The present paper describes pathotoxic fungal culture filtrates (FCF) as screening agent to select plant regeneration and analysis of antioxidant enzyme activities along with their isoenzyme patterns and chitinase activity on agar plate method in *Alternaria carthami* FCF-tolerant plants regenerated. A method for detection of chitinase activity on chitin agar plate after polyacrylamide gel electrophoresis using different staining dyes such as calcofluor white M2R, ruthenium red and Congo red were separately incorporated in chitin agar plates. After running polyacrylamide gel electrophoresis, the gel was transferred onto chitin agar plate containing different dyes for the activity staining. Thin layer of acetate buffer (0.2 M, pH 5) was poured on the gel, which helps faster diffusion of the enzyme from gel onto the plate. After incubation of about 7 h, bands of chitinase were visible by daylight or UV light. The method is very sensitive since it can detect even 0.5 units of chitinase. Thus, this method is sensitive, rapid, user-friendly, reliable and cost effective activity staining for chitinase detection in native and denatured Polyacrylamide gel. This study also examined antioxidant enzyme activity in FCF-tolerant plants. Catalase (CAT) activity was slightly decreased whereas peroxides (POD) activity was increased to a maximum of 48% from control and 57% from FCF-tolerant plants. Superoxide dismutase (SOD) activity was also increased to 37% and 49.5% in FCF-tolerant plants derived from leaf explants when compared with control plants. This method is more sensitive, rapid, reliable and cost effective than other mode of native and denatured Polyacrylamide gel methods.

Abbreviations: Superoxide Dismutase (SOD), Peroxidise (POD), Catalase (CAT), Ascorbate Peroxidase (APX) and Glutathione Reductase (GR), Fungal culture filtrates (FCF), PR (Pathogenesis-Related) Proteins.

Key words: *Asteraceae* · Fungal culture filtrates · Hygromycin · Glutathione reductase · Pathogenesis-Related Proteins

INTRODUCTION

Safflower, *Carthamus tinctorius* L., is a member of the family *Asteraceae*, cultivated mainly for its seed, which is used as edible oil. This crop was also grown for its flowers, used for colouring and flavouring foods and making dyes. Safflower is a highly branched, herbaceous, thistle-like annual, usually with many long sharp spines on the leaves. Fungal diseases have been one of the principal causes of crop losses ever since humans started to cultivate plants. Although breeders have succeeded in producing cultivars resistant to fungal diseases, the time-consuming processes of making crosses and back-crosses and the selection of progenies for the presence of resistance traits make it difficult to react adequately to the evolution of new virulent fungal races. Therefore, farmers often have to use chemicals. However, agrochemicals are costly and eventually they become less efficient due to the evolution of the pathogen [1,2] also developed Centennial and Morlin cultivars, with moderate resistance to *Alternaria* leaf spot and Pseudomonas bacterial blight.

Currently, much energy is being applied to identifying and isolating genes that upon transfer may render target plants resistant to fungi. Some of these efforts are being focused on resistance genes known from conventional breeding programs. Furthermore, there is an extensive search for genes that encode enzymes involved in the synthesis of compounds toxic to fungi and for genes that encode proteins with a direct inhibitory effect

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on the growth of fungi. Moreover, possibilities to exploit genes encoding inhibitors of fungal enzymes to obtain resistance are being investigated. Several strategies have emerged for developing crop varieties resistant to pathogens. Strategies include the manipulation of resistance by expression of PR (Pathogenesis-Related) proteins, antifungal peptides and manipulation of biosynthesis of phytoalexins. Fungal culture filtrates (FCF) and purified toxins have been used for *in vitro* selection and regeneration of disease resistant plants in many crops [3,4,5]. Recently, the fungal culture filtrates (FCF) have been identified as useful agents for the induction and selection of disease resistant mutants. There is currently great interest in the mechanism and the signal transduction pathway responsible for the gene expression induced by oxidative stress. In this case, the oxidative stress could be induced by FCF-tolerant plants. The oxidative stress is characterized by the activation and deactivation of many antioxidant defense enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) [6,7].

Chitinases (EC 3.2.1.14), i.e., enzymes hydrolyzing chitin, a persistent polymer of N-acetyl-D-glucosamine, attract researchers' interest due to their possible application in practice. Chitinases are considered to be potential fungicides, since they can hydrolyze chitin in the cell wall of phytopathogenic fungi [8]. One of the great interests is the use of chitinases for obtaining the degradation products of chitin and its deacylated derivative chitosan, which are widely used in biotechnology, agriculture, medical and cosmetic practices, etc. A great deal of interest has been generated on chitinase because of its applications in the biocontrol of plant pathogenic fungi [9], molting process of insects, mosquito control [10], production of chito oligosaccharide [11], single cell protein [12] and mycolytic enzyme preparation [13].

Glycol chitin is a soluble modified form of chitin, which has recently become a very useful substrate for activity staining but it is costlier than acid swollen chitin. Despite the importance of chitin metabolism in nature and *in situ* gel activity staining technique [14], there is still no method available for detection of chitinase activity onto the solid plate method after polyacrylamide gel electrophoresis under native or denaturing conditions. Chitin agar plate has been used earlier for isolating chitinolytic microorganisms and observing clear zone around the colony of microorganisms [15,16]. Attempts were therefore made to separate the crude chitinase on the polyacrylamide gel and transferred it onto the chitin agar plate.

MATERIAL AND METHODS

Plant Material and Explants Preparation: Safflower (*Carthamus tinctorius* L.) seeds (cv. NARI-6) sterilization procedures were performed as described by [17] and seed was aseptically germinated in MSG basal medium [18]. The Experimental study was conducted during July 2009 to October 2010. All the cultures were maintained at 25 ± 2°C under continuous irradiation with a white ?uorescent tube (30 μmol $m^{-2}s^{-1}$) for a photoperiod of 16 h light per day. Leaf explants excised from *in vitro* seedlings of both control and FCF-treated explants were taken for the present study.

Preparation of Fungal Culture Filtrates: Pathotype of *Alternaria carthami*, a virulent strain was obtained from Directorate of Oilseeds Research, Rajendranagar, Hyderabad, India. The fungal spores were initially cultured in petri dishes containing PDA (potato dextrose agar) solid medium and were incubated at 25°C. Fungal colonies (mycelium with spores) that appeared 7 days after plating on PDA media were transferred to PDA containing antibiotics selection medium (100 μg/ml hygromycin B) [19]. After 10 days, colony growth and morphology were observed. Mycelia mats with spores were collected from the petri dishes and inoculated directly into Erlenmeyer flasks, containing MS basal liquid medium for culture filtrate production [20]. The various levels of culture filtrates ranging from 10-50 % (v/v) were mixed with above regeneration medium.

Extraction of Soluble Protein, Antioxidant Enzyme Assays: Extraction of Soluble Protein was described by [21] Pradeep Kumar *et al.* 2011. Protein content was determined by Bradford method [22]. CAT activity was measured according to [23]with some modifications. 50mM potassium phosphate buffer, pH 7.0 containing 20 mM H_2O_2 were prepared for CAT activity at 25°C by monitoring the consumption of H_2O_2 at 240 nm (extinction coefficient 39.6 mM^{-1} cm^{-1}) for 5 min and the changes in absorbance every 20s.

POD activity was determined specifically at 25°C with guaiacol at 470nm following the method of [24]. The reaction mixture contained 40mM potassium phosphate buffer (pH 6.9), 1.5 mM guaiacol and 6.5 mM H_2O_2 in a 3-ml volume. SOD activity was determined as described by [25]. The enzyme activity was performed at 25°C in a 3-ml volume containing 50 mM Na_2CO_3:$NaHCO_3$ buffer (pH 10.2), 0.1 mM EDTA, 0.01 mM ferricytochrome C and 0.05 mM xanthine. The absorbance was measured at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}).

Native PAGE Activity: Equal amount of protein leaf samples (10 µg) from control and FCF-tolerant plants were taken for each experiment. Gel electrophoresis was performed in 10 % non-denaturing polyacrylamide gels at 4°C for 1.5 h with a constant current of 30 mA after loading equal amounts of protein from control and FCF-tolerant plants. After electrophoresis, the gels were stained for the enzymatic activities. Catalase activity was determined by incubating the gels in 3.27 mM H_2O_2 for 25 min, rinsed in water and stained with 1% potassium ferricyanide and 1% ferric chloride for 4 min [26]. Staining of POD was determined by incubating the gels in sodium citrate buffer (pH 5.0) containing 9.25 mM p-phenylene diamine and 3.92 mM H_2O_2 for 15 min [27]. Gels were stained for SOD by soaking in 50 mM potassium phosphate, pH 7.8, containing 2.5 mM photochemical nitroblue tetrazolium (NBT) in darkness for 25 min, followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 28 mM nitroblue tetrazolium and 28 mM riboflavin in darkness for 30 min [28].

Enzymatic Assay of Chitinase: Chitinase activity was assayed by measuring the amount of reducing sugars produced from chitin. The reducing sugars were determined with dinitrosalicylic acid (DNS) [29]. The reaction mixture for chitinase assay contained 0.5 ml of 0.1 M potassium phosphate buffer (pH 6.75), 0.4 ml of 12 mg/ml colloidal chitin and 0.1 ml of the material tested. The mixture was incubated at 50°c for 30 min. One unit of activity was defined as the amount of enzyme liberating 1 mg of N-acetyl-D-glucosamine per hour.

Chemicals Required: Practical grade chitin, glycol chitin, calcofluor white M2R were obtained from Sigma Chemical Co. (St. Louis, MO). Congo red was obtained from BDH (Poole, England). Ruthenium red was obtained from HiMedia Laboratories Ltd. (Mumbai, India).

SDS-PAGE (Polyacrylamide Gel Electrophoresis): The electrophoretically separated proteins were denatured and their chitinase activity was determined with the ?uorescent dye as described by [14]. Native and SDS polyacrylamide gel electrophoresis were carried out at a constant current of 20 mA in 12% (w/v) gels (1.5 mm thick) by method of [30]. The gels were run at 4°C. After SDS electrophoresis, gels were incubated at 37°C for 4 h in sodium acetate buffer (0.2 M, pH 5) containing 1% (v/v) Triton X-100 to remove SDS. The gels were washed with distilled water.

Staining for Chitinase Activity: Preparation of chitin agar plate with different dyes Chitin agar plates were prepared using 0.5% acid swollen chitin or 0.5% glycol chitin [31]. Gels after native and SDS PAGE were directly transferred onto each chitin agar plate containing a different dye. Thin layer of acetate buffer (0.2 M, pH 5) was put on each gel for diffusion of chitinase onto the chitin agar. The plates were incubated at 37°C. Activity band is visible as formation of dark band against a fluorescent background on chitin agar plate, with calcofluor white M2R, after 7 h. This was observed under a hand held UV transilluminator (UVP Inc., Upland, CA, USA).Whereas chitin agar plates containing ruthenium red and Congo red showed clear distinct bands on the plate observed in daylight. This method is a simple, reproducible, sensitive, user-friendly, reliable and cost effective activity staining for chitinase detection in native and denatured polyacrylamide gel.

RESULTS

Responses of Antioxidant Enzyme Activity: The activities of antioxidant enzymes participating in the scavenging of oxidative stress were assessed in control and FCF-tolerant leaf samples. CAT activity was slightly decreased in 40 % FCF-tolerant leaf samples, where as POD and SOD activities were greater when compared with control plants. POD activity was increased to maximum of 48 % (0.19 µmol min^{-1} mg^{-1} protein) and 57 % (0.15 µmol min^{-1} mg^{-1} protein) in FCF-tolerant plants. SOD activity was also increased to 37 % (145 U mg^{-1} protein) and 49.5 % (140 U mg^{-1} protein) in FCF-tolerant plants over control experiment (Table 1).

Table 1: Antioxidant enzyme activities on FCF-tolerant leaf explants and control leaf explants of saf?ower (*Carthamus tinctorius* L.)

	Control Leaf Explants			FCF Treated Leaf Explant		
FCF Conc. (%)	CAT	POD	SOD	CAT	POD	SOD
0	1.9 ± 0.9a	1.05 ±1.5c	1.19 ±1.1d	1.94 ±0.1a	1.20 ±0.01c	1.23 ±3.2bc
10	2.25 ± 1.5ab	1.59 ± 1.9bc	2.32 ±4.0c	2.50 ±0.5ab	1.71 ± 0.05bc	2.67 ± 1.0b
20	2.40 ± 1.9b	2.13 ± 2.5b	2.90± 2.1b	2.60 ± 0.2b	2.35 ± 0.01b	3.10 ±6.8ab
30	2.22 ± 1.6bc	2.15 ± 1.9ab	2.26± 1.0	2.56 ± 0.1c	2.30 ± 0.03ab	3.20 ±5.0ab
40	1.06 ± 0.7c (23%)	1.19 ± 1.1a(48%)	1.22 ±4.2a(37%)	2.03 ± 0.9c(30.7%)	1.53 ± 0.02a(57%)	2.95 ± 1.7a (49.5%)

CAT (µmol O_2 $min^{-1}mg^{-1}$protein), POD (µmol $min^{-1}mg^{-1}$protein) and SOD (U mg^{-1}protein)

Values are mean ± SE of three repeated experiments. Means within a column followed by the same letters are signi?cantly different according to ANOVA and Duncan's multiple range test (P>0.05). Values are mean ± SE of three repeated experiments.

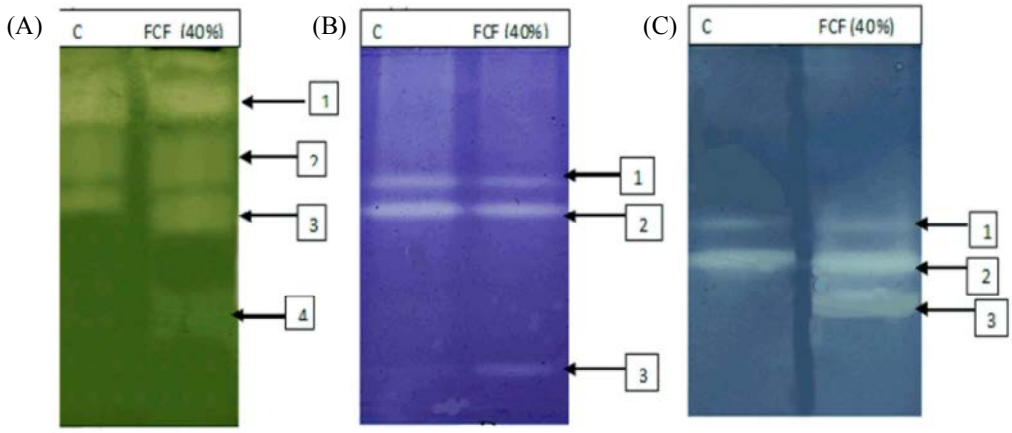

Fig. 1: Staining activity of antioxidant enzymes in safflower (*Carthamus tinctorius* L.)
A-CAT-catalase activity in control and FCF-tolerant leaf samples.
B-POD-peroxidase activity in control and FCF-tolerant leaf samples.
C-SOD-superoxide dismutase activity in control and FCF-tolerant leaf samples.
(C-control, FCF-fungal culture filtrates)

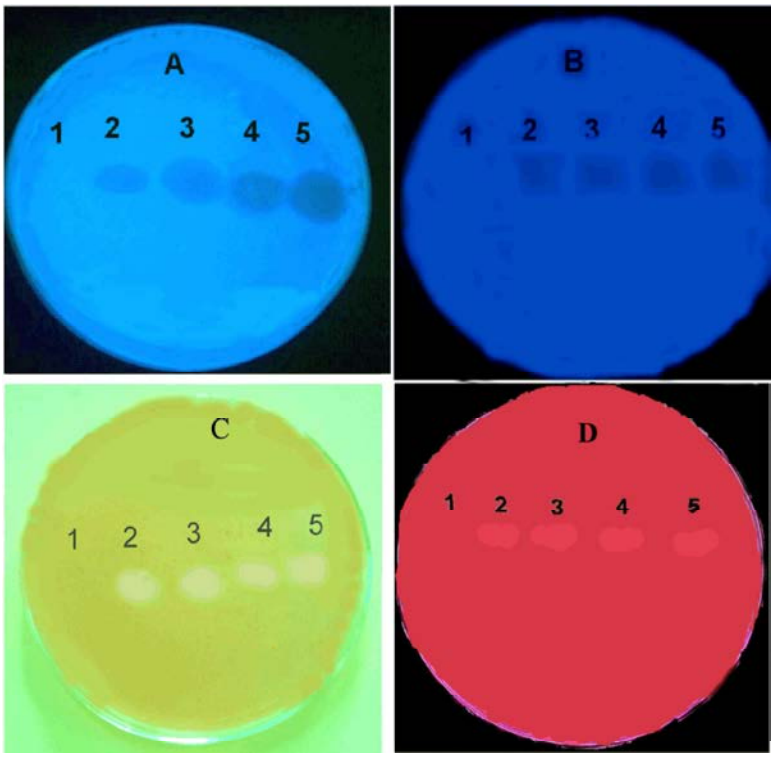

Fig. 2: Chitinase activity staining of electrophoretically resolved native protein of chitin by solid plate method, containing A) glycol chitin with calcofluor white M2R (control); B) glycol chitin with calcofluor white M2R(FCF-treated); C) acid swollen chitin with ruthenium red; D) acid swollen chitin with Congo red; Different units of chitinase 1.(0.5); 2.(0.9); 3.(5.0); 4. (8.0); and 5. (10.0) were loaded onto the gel to study the sensitivity.

The isoforms of different enzymes was analyzed by native PAGE. There were no much differences in banding pattern for CAT, POD and SOD. Gels stained for CAT revealed two thick bands in control and three thick bands and banding size was increased in FCF-tolerant leaf samples (Fig. 1A). POD and SOD exist as multiple isoforms in control and FCF-tolerant plants (Fig.1B and 1C). In control plants, (2 and 3) bands of POD were

detected and the staining intensity was increased. However, 3 bands were induced from FCF-tolerant plants and had a quite strong staining intensity. For SOD activity, 3 isoforms from control plants and 3 isoforms from FCF-tolerant plants were observed and their staining intensity was also increased. The induction of new fourth isoforms from both POD and SOD was quite FCF specific (Fig.1B and 1C).

Chitinase Assay: Chitinase in both SDS and native PAGE, the concentrations of chitinase (0.5 to 10 units) were loaded. After electrophoresis, native gels were transferred onto the plate containing acid swollen chitin and glycol chitin with calcofluor white. For SDS PAGE, chitinase was renatured by removing SDS using triton X 100 (0.5% v/v) treatments. The acetate buffer was layered onto the gel. The diffusion of chitinase onto plate was measured on the basis of the band observed. Without acetate buffer (0.2 M, pH 5), the time taken for diffusion was found to be 13 h whereas with acetate buffer the time taken for diffusion decreased by 6 h thus indicating an increase in diffusion rate. In this method other than calcofluor white M2R, ruthenium red and Congo red [0.001% (w/v)] were used with acid swollen chitin as a substrate in the plates. Plates incorporated with Congo red and ruthenium red did not show significant improvement in Visualization of distinct band as compared to ruthenium red and Congo red. Whereas calcofluor white M2R shows very good distinct bands on solid plates when compared to others (Figure 2).

DISCUSSION

The present research was focused on the 40% *Alternaria carthami* disease resistant safflower (*Carthamus tinctorius* L.) leaf explants were used for the analysis of antioxidant enzyme activities along with their isoenzyme patterns *in Alternaria carthami* FCF-tolerant plants. *In vitro* selection for resistance to a pathogen can be carried out when *in vitro* cultures are exposed to toxins produced by the pathogen, or synthetic toxin analogues to a pathogen filtrate, or extracts of the pathogen, or to the pathogen itself [32]. Embryogenic callus tissues can be exposed easily and uniformly to fungal culture filtrates produced by *A. carthami* fungi for resistant calli production. The frequency in the production of resistant calli was decreased with increasing concentration of toxins in the medium. In the present study, the toxin resistant (insensitive) leaf was used for Antioxidant enzymes studies and chitinase activity from both control and FCF-treated leaf samples.

Antioxidant enzymes are likely to play a considerable part of defense mechanism against FCF induced oxidative stress. CAT involved in an efficient protective role to oxidative stress [33].The increase in the activities of POD, SOD might occur against oxidative stress or serve to compensate for low CAT activity. So CAT and peroxidases inductions appeared to be a common response to stress that could strengthen plants against reactive oxygen species overproduction. The activities of POD and SOD considerably increased in FCF when compared to control plants. In this case, FCF-treated plants were capable of synthesizing new isoforms of POD and SOD, which could be considered as a response of FCF induced oxidative damage. Moreover, chitinase are pathogenesis related protein (PR) exist in different forms in plants and may be somehow involved in host defense against fungal pathogens [34, 35]. Finally, FCF on plants increased enzyme activity that could result in an increased effect on the defence of safflower (*Carthamus tinctorius* L.) against fungal attack.

We developed techniques for screening of hyper chitinase using acid swollen chitin with calcofluor white M2R in plate assay. Dark haloes were observed around samples against fluorescent background under UV light [36].The same approach was tested for the detection of chitinase activity on solid agar plate through gel electrophoresis.[14] have developed an activity staining method by incorporating a soluble glycol chitin in electrophoresis gel. Lytic zone was observed by UV illumination with a transilluminator after staining with calcofluor white M2R.In this method, when substrate was directly incorporated into gel the bands showed a smear instead of well-defined band. We observed retardation of mobility of enzymes in the gel during the electrophoresis, which may be because of the presence of polysaccharide in the gel, which can be overcome by solid plate method. As acid swollen chitin costs lesser than glycol chitin, this method is also cost effective in terms of the substrate. Different concentrations of chitinase were used in order to determine the sensitivity. The method is very sensitive since it can detect even 0.5 units of chitinase.[37] reported sensitivity of in situ activity staining method of 10 units when casein was used in the gel wash buffer. Calcofluor white M2R binds to the glucan chains and linear b-(1,4)-glucosidically linked units of N-acetyl glucosamine. On binding to polysaccharide such as cellulose and chitin, this flourochrome highlights and emits a light blue light when exposed to UV. On degradation of this polymer to it individual subunits, this fluorescence is lost as indicated by a dark band against a fluorescent

background. Calcofluor white M2R is widely used as an optical brightener in the textile industry and also as a staining dye in fluorescence microscopy [38] and for the screening of hyperchitinase producing bacteria. Calcofluor white M2R incorporated chitin plate is suitable for sensitive plate assays or semi-quantitative enzyme diffusion plate assay [36]. The chitinase method we have developed is more sensitive, rapid, user friendly and reliable for safflower plant samples.

CONCLUSION

In response to the pathotoxic fungal culture filtrates (FCF) as screening agent to select plant regeneration and analysis of antioxidant enzyme activities along with their isoenzyme patterns and chitinase activity on agar plate method in *Alternaria carthami* FCF-tolerant plants regenerated. A method for detection of chitinase activity on chitin agar plate after polyacrylamide gel electrophoresis using different staining dyes were used. This study also examined antioxidant enzyme activity in FCF-tolerant plants. Catalase (CAT) activity was slightly decreased whereas peroxides (POD) activity was increased to a maximum from FCF-tolerant plants than control plants. Superoxide dismutase (SOD) activity was also increased in FCF-tolerant plants than control.

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