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Research article

Evaluation of rust disease tolerance and antioxidant enzyme activity from organogenesis and somatic embryogenesis in safflower (*Carthamus tinctorius* L.) cv. NARI-H-15

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Abstract

Importance of the work: Rust fungi (*Puccinia carthami*) play a critical role as major threats to diminishing safflower growth, quality, and productivity worldwide. Therefore, there is a need to develop and deploy rust-resistant safflower plants. Recently, *in vitro* selection of fungal toxins has been an effective tool for disease resistance in many crops.

Objectives: The present study aimed to standardize an efficient protocol for rust-resistant safflower (*Carthamus tinctorius* L.) cv. NARI-H-15 plant development through organogenesis and embryogenesis processes using *P. carthami* fungal culture filtrates (FCFs) as selection pressure.

Materials & Methods: *In vitro* FCF selection technique with organogenesis and somatic embryogenesis pathways were used for the experimental purpose to develop tolerant plants. Antioxidant enzyme and pathogenicity assay methods were applied to determine the level of tolerance against biotic stress.

Results: For organogenesis, immature leaf calli cultured on 40% FCF-selected Murashige and Skoog (MS) medium induced 22.8% more shoot organogenesis and 16.6% more rooting than cotyledonary leaf calli. For somatic embryogenesis, cotyledon-derived embryogenic calli cultured on 40% FCF-selected MS medium produced 30% more somatic embryogenesis with 17% more germination than immature-leaf-derived embryogenic calli. Fully regenerated FCF-tolerant plants were transferred to pots containing a soil mixture, with 52% of plants survived under greenhouse conditions. Further experiments evaluated the reactive oxygen species scavenging enzymes in FCF-tolerant plants. Catalase activity decreased while peroxidase and superoxide dismutase activities were higher in the FCF-tolerant plants than for the control, based on quantitative and qualitative analysis. The pathogenicity results confirmed the disease resistance of the *in vitro*-developed FCF-treated plants.

Main finding: Application of 40% FCF in tissue culture medium appears to be the reliable strategy for enhancing rust disease resistance in safflower cv. NARI-H-15 against *P. carthami.*

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Introduction

Safflower (*Carthamus tinctorius* L.) is one of humanity's oldest crops (Singh and Nimbkar, 2016), belonging to the family Asteraceae. It can grow well in various types of soil and involving to the sustainable agricultural production and employment (Akgun and Soylemez, 2022). Safflower has many health benefits and possesses many pharmacological activities (Mani et al., 2020). It is cultivated mainly in India for its oil-rich edible seed and its flowers are used traditionally for coloring foods and medicines. According to Gyulai (1996), safflower is a minor crop with commercial seed production of approximately 800,000 tonnes per year. Seeds have been produced commercially as a source of edible oil used in cooking, margarine and salad dressing (Popov and Kang, 2011). Many cultivars of safflower are affected by serious blight, rot, rust, and wilt diseases, with more localized foliar diseases being caused by *Alternaria carthami*, *Cercospora carthami, Puccinia carthami* and *Ramularia carthami* (Dajue and Mündel, 1996). Foliar, stem and root infections start from seedling infections by germination of aeciospores or urediospores. The lower leaf surface is infected with *P. carthami* and develops small, powdery, and chestnut-brown pustules abundantly. A pronounced girdling and hypertrophy of the stem base may occur with rust disease. These plants very often break at the girdled areas due to heavy wind and rainstorms (Nyvall, 1989). Infection of safflower seedlings starts from soil-borne and seed-borne teliospores, and then the aeciospores are wind-blown to the safflower plants to cause uredial infections with uredospores. The drooping, wilting, and hypertrophied hypocotyls occur by primary seed infection. Cotyledons with pustules appear to result in pycnidia and aecia (Prasada and Cothia, 1950). The development of resistant safflower varieties may control the systemic spread of rust disease. Most probably, the fungicide treatment can preclude the rust disease, but such treatment does not protect young seedlings from infection by soil-borne spores of *P. carthami* (Cappelli and Zazzerini, 1988).

Susceptibility to rust disease is a major limiting factor for safflower plant production in India. Plant breeding for disease resistance in safflower may fail because every cultivar is highly susceptible to rust infection. Recently, safflower cv. NARI-H-15 was developed as the spiny variety which is early maturing and tolerant to aphids and wilt (Singh and Nimbkar, 2018), but it is highly susceptible to rust infection (Dajue and Mündel, 1996). Infection caused by pathogens may result in major economic loss at the global level. Traditional methods have failed to produce the desired crop improvement outcomes against pathogens (Gupta and Acharya, 2018). *In vitro* tissue culture is an important plant propagation method that can be exploited as a tool in safflower improvement programs. There are many published reports on the regeneration of safflower plants (e.g., Vijayakumar et al., 2008a; Vijayakumar et al., 2017; Surbhaiyya et al., 2018; Mendhe and Sheikh, 2018; Varpe and Mendhe, 2021).

Improved biotechnological tools such as transgenic techniques (Tohidfar and Khosravi, 2015) and elicitors (Chandra et al., 2015, 2017) are highly applicable for solving many vital problems in crop plants. *In vitro* selection of fungal toxins has emerged as an underlying process for regenerating resistant plants, as it is an ideal method for understanding the disease resistance at the cellular, subcellular (Jayasankar et al., 2000) and molecular (Jayasankar and Gray, 2004) levels. Pathotoxins seem to have the capacity to change the accumulation pattern of primary and secondary metabolites (Wojakowska et al., 2013; Rojas et al., 2014), defensive compounds (Gupta et al., 2013; Gupta and Acharya, 2015, 2016), hypersensitive responses (Kombrink and Schmelzer, 2001) and systemic acquired resistance and induced systemic resistance (ISR; Cipollini et al., 2004). Disease resistance can be attained by applying selection agents such as fungal toxins in the tissue culture media. *In vitro* selection pressure with pathotoxins can permit the survival of a few cells from explants for the regeneration of plants. The appearance of desirable resistance traits in *in vitro*-developed plants can be genetically stable in crop improvement (Gupta and Acharya, 2018). An *in vitro* selection strategy is viable for attaining resistance in many crop plants through the expression of pathogenesis-related proteins, antifungal peptides, biosynthesis of phytoalexins, fungal culture filtrates (FCF) and purified toxins (Lynch et al. 1991; Ahmed et al. 1991; Gupta and Acharya, 2018). However, *in vitro* selection of fungal culture filtrates is a possible solution to resolve the recent challenges.

These characteristics have attracted many researchers to apply fungal toxins as a selective agent for achieving disease resistance using an *in vitro* approach. Vijayakumar et al. (2008b) selected pathotoxic FCFs to regenerate *Alternaria carthami* fungal-resistant safflower cv. NARI-6 plants through organogenesis and somatic embryogenesis. The present study describes pathotoxic FCFs as screening agents to select plant regenerants through organogenesis and somatic embryogenesis. The efficient protocols for organogenesis and somatic embryogenesis are necessary to regenerate rust

disease-resistant safflower cv. NARI-H-15 plants through *in vitro* selection of *P. carthami* FCFs.

Materials and Methods

Plant Materials

Seeds of safflower cv. NARI-H-15 were procured from the Nimbhkar Agricultural Research Institute (NARI), Maharashtra, India. This cultivar seed was selected based on the area of cultivation, agro-climatic conditions and susceptibility to prevalent rust fungal diseases. Seed sterilization procedures were performed as described by Vijayakumar et al. (2017). The sterilized seeds were germinated aseptically on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) comprised of 3.0% sucrose and 0.8% agar. All cultures were incubated under continuous irradiation at 25 ± 2 °C for a 16 hr photoperiod using a cool white fluorescent tube (15 µmol/m^2/s^1) and 7–9 day-old cotyledonary leaf and immature leaf explants were used for the present study.

Preparation of P. carthami fungal culture filtrates

The rust-diseased portion on the cotyledon, leaves and girdling of the stem was excised from the young seedling stages of safflower cv. NARI-H-15 using a sterile surgical blade. They were soaked with 2–3 drops of 10% Teepol solution and kept under running tap water for 10–15 min. The surface-sterilized rust-diseased pieces were treated with 0.1% mercuric chloride solution for 2 min and cultured on Petri dishes containing potato dextrose agar (PDA) medium and incubated at 27 °C. Mycelia with spores of *P. carthami* fungal strains appeared 5 d after plating on PDA. The formations of colonies of fungal mycelium with spores on the surface of the infected portions of cotyledon, leaves and stem were isolated using an inoculation loop and subcultured on PDA medium containing antibiotics (100µg/ml hygromycin B), according to Hatta et al. (2002). Sporulated mycelial growth was observed under a microscope after 7 d and preserved at 4 °C. The sporulated mycelial mats were peeled from the Petri dishes (Fig. 1A) and inoculated in Erlenmeyer flasks containing MS basal salts (Murashige and Skoog, 1962) and 3.0% (weight per volume; w/v) sucrose medium for culture filtrate production (Vijayakumar et al., 2008b). All cultures were maintained in a shaker at 100 revolutions per minute (rpm) under dark conditions. After 30 d incubation, the reddish, dark brown culture filtrate that had synthesized in the medium (Fig. 1B) was passed through Whatman No.1 filter paper to separate the fungal spores. The filtrate was centrifuged at 3,000 rpm for 15 min. The supernatant of the filtrate was finally separated by passing through filter paper. Then, the filtrates were collected in brown storage bottles and kept in a laboratory refrigerator (-20 °C). The volume of filter-sterilized FCFs ranging from 10% to 70% was mixed with organogenesis and somatic embryogenesis media

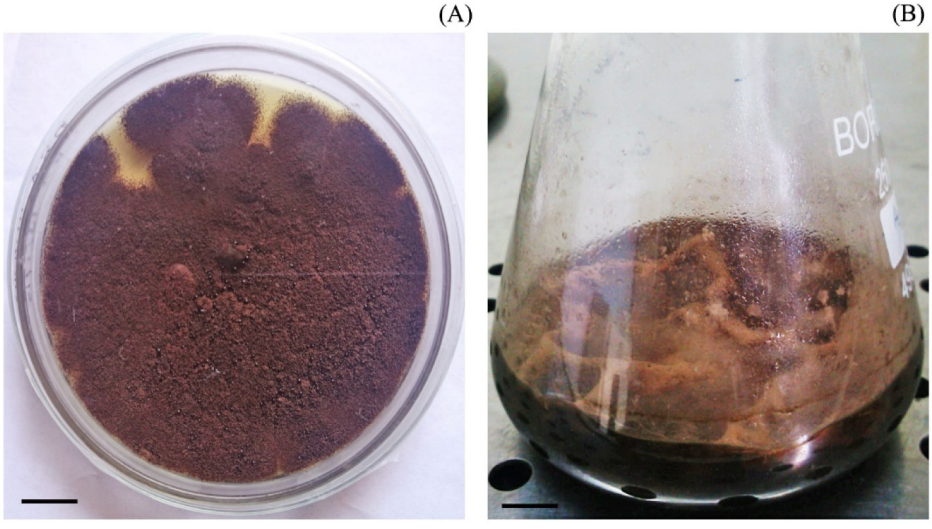

Fig. 1 Figures showing *Puccinia carthami* fungal culture filtrate: (A) pure culture of *P. carthami* in potato dextrose agar medium after 7 d (scale bar = 4 mm); (B) fungal mycelium with fungal culture filtrate production in Murashige and Skoog basal medium after 30 d (scale bar = 5 mm)

In vitro culture conditions

The required levels of organic and inorganic salts were thawed and added with sucrose (3.0%) and gelling agent (0.8% agar) to prepare MS basal medium. The medium was supplemented with FCF at different concentrations (0–70%). Before the addition of agar to the medium, the pH of the medium was adjusted to 5.7 using 0.1N solution of either HCl or NaOH. Then, the mixture was boiled to melt the agar and about 10–15 mL of the solutions were dispensed to the culture vials (25 mm × 150 mm) or 30–50 mL of solution was transferred to 250 ml Erlenmeyer flasks and closed with a cotton plug. Culture vials with media were autoclaved at 121 °C for 15 min. All culture tubes were kept inside the culture room to allow for solidification and cooling of the medium.

Induction and proliferation of fungal culture filtrate-resistant organogenic calli

Seven-day-old cotyledonary leaf and immature leaf explants (0.5–1.5 cm long) were isolated from *in vitro* seedling plants of safflower cv. NARI-H-15 and subcultured on MS basal salts, 0–70% FCF (volume per volume; v/v), 3.0% sucrose (w/v), 0.8% agar (w/v) medium fortified with 1.5 mg/L N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU; Vijayakumar et al., 2017). All cultures were incubated at 25±2 °C for a 16 hr light and 8 hr darkness period using a white fluorescent tube (15 µmol/m^2/s) for FCF-resistant calli induction. These resistant calli were collected from the selective medium and subcultured on fresh medium containing the same composition to enhance proliferation after 45 d of culture. The experiment was carried out with 35 explants and was repeated three times. The callus induction frequency was defined as the percentage of explants producing callus. The mean fresh weight and dry weight of the callus were measured after 60 d of culture initiation.

Regeneration of fungal culture filtrate-resistant plants via organogenesis

Approximately 100 mg of FCF-resistant calli were separated from cotyledonary leaf and immature leaf explants and inoculated on MS basal salts, 0–50% FCF, 3.0% sucrose (w/v), 0.8% agar medium supplemented with 4.0 mg/L mT (meta-Topolin) + 1.5 mg/L CPPU (Vijayakumar et al., 2017). All cultures were maintained at 25±2 °C for a 16 hr light and 8 hr darkness period using a white fluorescent tube (15 µmol/m^2/s) for adventitious microshoot regeneration. Each treatment was performed with 40 explants and the experiments were repeated three times. Data on the percentage of FCF-tolerant shoot organogenesis with the development of the mean number of microshoots were recorded after 55 d of culture. Individual microshoots (0.5–1.5 cm height) were isolated from the FCF resistant calli using a surgical blade and subcultured on rooting medium comprised of quarter strength MS salts, 0–50% FCF, 1.0% sucrose, 0.8% agar with the required level of 1-naphthaleneacetic acid (NAA; 1.5 mg/L) + mT (0.1 mg/L) (Vijayakumar et al., 2017) for adventitious root induction. Data were recorded on the percentage of root induction after 30 d of culture.

Induction and proliferation of fungal culture filtrate-resistant embryogenic calli

The 7–9 day-old cotyledonary leaf and immature leaf explants (0.5–1.5 cm long) were excised from *in vitro* seedlings and inoculated on MS basal medium comprised of FCF ranging from 0% to 70% (v/v), 3.0% sucrose (w/v) and 0.8% agar (w/v) along with the required level of mT (1.5 mg/L) + picloram (3.0 mg/L) + CPPU (1.0 mg/L). All cultures were incubated at 25 ± 2 °C for a 16 hr light and 8 hr darkness period using a white fluorescent tube (15 µmol/m^2/s) for FCF toxic-insensitive calli induction. Each subculture was made after a 2 wk interval on fresh FCF selective medium for the proliferation of FCF-resistant embryogenic calli. Each experiment consisted of 40 explants and was replicated three times. The FCF-resistant embryogenic callusing frequency was defined as the percentage of explants that produced callus per culture. The mean fresh weight of FCF-resistant embryogenic callus was determined after 60 d of culture.

Effect of fungal culture filtrate on somatic embryogenesis

Proembryogenic callus tissue (approximately 100 mg fresh mass) was collected from cotyledonary leaf and immature leaf explants and inoculated on half-strength MS basal salts, 1.5% sucrose (w/v), 0.8% agar (w/v), 0–50% FCF (v/v) medium consisting of the required level of polyamines (20 µM putrescine + 15 µM spermidine) and mT (4.0 mg/L) + picloram (1.0 mg/L) + CPPU (0.3 mg/L) for the different stages of somatic embryo induction. The different stages of embryos on embryogenic callus tissues were subcultured on liquid medium using various concentrations (0–50%) of toxic FCF. All cultures were incubated at 25 ± 2 °C on a rotary shaker

at 100 rpm and exposed to 15 µmol/m^2/s of light provided by a white fluorescent tube for 16 hr light and 8 hr darkness photoperiod to proliferate somatic embryos. There were five flasks per experiment and each experiment was repeated three times. Data were collected in terms of the percentage of embryogenesis with average somatic embryo formation measured after 45 d of culture. The embryos on liquid cultures were sieved and subcultured on quarter-strength MS basal salts, 0–50% FCF, 1.0% sucrose, 0.8% agar, myoinositol (50 mg/L), thiamine HCl (3.0 mg/L) medium included with 0.3 mg/L gibberellic acid (GA_3) and 0.1 mg/L mT for maturation and germination. The germinated embryos with or without root poles were subcultured on quarter-strength MS basal salts, 1.0% sucrose, 0.8% agar medium fortified with NAA (1.5 mg/L) + mT (0.1 mg/L) for root induction.

Hardening of fungal culture filtrate-tolerant plants

The rooted plants from the organogenesis and germinated cotyledonary stage embryos with shoot poles and root poles from somatic embryogenesis were carefully isolated from the culture vessels and rinsed off in tap water to remove agar gel and then implanted in plastic cups consisting of sterile red soil, garden soil and sand mixture in the ratio of 1:2:1. The plastic cups were covered with polythene bags with holes in them to control the high humidity. The potted plants were irrigated with 5 mL of autoclaved half-strength MS basal medium prepared with distilled water during the first 2 wk. The polythene bags were removed out and the surviving plants were transplanted to earthen pots containing soil mixture, and subsequently to a field greenhouse.

Extraction of protein and antioxidant enzyme assays

Samples (1.0 g) of fresh leaf were isolated from the control and FCF-tolerant plants regenerated through organogenesis and embryogenesis, homogenized in 2 mL buffer containing 50 mM potassium phosphate (pH 7.5) and 2 mM tris ethylene-diamine-tetraacetic acid (EDTA). The homogenate fractions were separated using centrifugation (15,000×*g*) at 4 °C for 15–20 min. The protein in the supernatant was concentrated to enhance the stability of the actual conformation using ammonium sulfate precipitation (Wingfield, 2001) and passed through Whatman No.1 filter paper. The activities of three enzymes were determined using a spectrophotometer and the total content of protein was determined according to Bradford (1976). The total peroxidase (POD) activity was estimated using guaiacol at 470 nm and 25°C (Egley et al., 1983). Each 1 mL of solution consisted of 1.5 mM guaiacol, 40 mM potassium phosphate buffer (pH 6.9) and 6.5 mM H_2O_2 in the reaction. The total catalase (CAT) activity was measured according to Piacentini et al. (2001) with some modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and 20 mM H_2O_2 for assessing the CAT activity at 25 °C in terms of H_2O_2 consumption (extinction coefficient = 39.6 /mM/cm) in absorbance at 240 nm per minute. The superoxide dismutase (SOD) activity was measured based on Spychalla and Desborough (1990). The reaction mixture (3 mL) was composed of 50 mM Na_2CO_3:$NaHCO_3$ buffer (pH 10.2), 0.1 mM EDTA, 0.05 mM xanthine and 0.01 mM ferricytochrome C. The level of SOD activity was recorded in the reaction mixture incubated at 25°C and based on the absorbance of the wavelength at 340 nm (extinction coefficient = 6.2/mM/cm).

Staining for antioxidant enzyme activity using native polyacrylamide gel electrophoresis

Extracted protein samples (each 15 µg) from the control and FCF-tolerant plants were used for enzymatic activity following separation using native polyacrylamide gel electrophoresis (PAGE). Initially, non-denaturing polyacrylamide gel was prepared using 10% polyacrylamide without sodium dodecyl sulfate. Equal amounts of protein extracted from the control and FCF-tolerant plants were loaded into corresponding gel wells. Electrophoresis was performed at 4 °C for 1.5 hr with 30 mA current supply after loading. After electrophoresis, the enzymes retained inside the native gels were subjected to specific activity staining. For POD staining activity, the gels were incubated in sodium citrate buffer (pH 5.0) consisting of 9.25 mM *p*-phenylenediamine and 3.92 mM H_2O_2 for 15 min (Olson and Varner, 1993). Catalase activity was observed from the gels incubated in a solution containing 3.27 mM H_2O_2 for 25 min and washed with deionized water; 1% potassium ferricyanide and 1% ferric chloride solution was prepared to improve staining for 4 min (Woodbury et al., 1971). According to Rao et al. (1996), gels were stained for SOD by soaking in 50 mM potassium phosphate (pH 7.8) buffer containing 2.5 mM photochemical nitroblue tetrazolium and kept in the dark for 20 min. The gel was immersed in 50 mM potassium phosphate (pH 7.8), 28 mM nitroblue tetrazolium and 28 mM riboflavin mixed solution in the dark for 30 min.

Pathogenicity assay

A virulent fungal strain (*P. carthami*) was inoculated on PDA medium for the production of spores. The reddish-brown colored spores were collected by washing the surface with distilled water. The spore suspension was obtained by filtering through cheesecloth to remove the entire mycelial blocks. The filtered spore suspension was concentrated using centrifugation at 2000×*g* for 5 min and washed with deionized sterile water to obtain clear spore pellets. A sample (1 g) of dried spore pellets was mixed with 100 mL deionized sterile water consisting of 0.1% Tween-20. Pathogenicity was assayed by spraying the conidial suspension (5×10^5 conidial spores/mL) on leaves and girdling the stem of the control and FCF-tolerant safflower cv. NARI-H-15 plants grown under greenhouse conditions. Data on the percentage survival of hardened plants from organogenesis and somatic embryogenesis were recorded every weekend. All plants were covered with polythene bags to prevent inadvertent spore dispersal. The number of rust color spots appeared on the leaves and diseased portions of the stem girdling from both the control and FCF-tolerant plants (T0) were measured every week.

Statistical analysis

All experiments were carried out using a complete randomized block design. Every treatment was replicated three times. Data were expressed as the mean ± SD for different variables in the independent experiments. The experimental data pertaining to FCF-resistant organogenic and embryogenic callus induction, shoot organogenesis, somatic embryogenesis per culture, antioxidant enzyme analysis and the pathogenicity studies were subjected to one-way analysis of variance followed by mean separation based on Duncan's multiple range test using the SPSS (version12.0) software. Statistical significance was set at $p < 0.05$.

Results and Discussion

Effect of fungal culture filtrate on plant regeneration via indirect organogenesis

The application of a fungal toxin *in vitro* was more advantageous for developing resistant plant cells (Daub, 1986). Generally, the fungal toxin or culture filtrate may contain low molecular weight peptides, terpenoids and carbohydrates (Huang, 2013). The incorporation of culture filtrates of pathogens into culture media can be a valuable source to demonstrate the strong inhibitory effect on the development of plants. The results of the present study observed the source of resistance in the organogenic callus produced from cotyledonary leaf and immature leaf explants on FCF of *P. carthami* selective medium. Exposure of the organogenic calli to the toxic medium resulted in a variable survival rate. Cotyledonary leaf- and immature leaf-derived organogenic calli survived well and were resistant to FCF. The FCF (0–70%) selective medium fortified with the optimum level of CPPU (1.5 mg/L) reduced the frequency of pathotoxic resistant calli for both cotyledonary leaf and immature leaf explants culture. The frequencies of induction of pathotoxic-resistant calli gradually decreased in all FCF selective medium. FCF at 40% showed slow growth and browning of the organogenic callus. The repeated subculture reduced the programmed cell death in the 40% FCF-treated callus tissues. Therefore, the toxic-insensitive calli production for cotyledon and immature leaf explants was optimized at 40% FCF selective medium, while programmed cell death was greater for the 50% FCF treatment. In this case, there was about 16.6% toxic-resistant callusing frequency with 54 mg fresh mass and 29 mg dry weight of organogenic calli formation from cotyledonary leaf explants cultured at 40% FCF compared to the control experiment. The immature leaf explants had a maximum of 18% toxic-insensitive callus induction frequency with an average of 66 mg fresh weight and 32 mg dry weight of calli after 60 d of culture initiation (Fig. 2A and 2B; Fig. 3A and 3B). In the 60–70% FCF treatments, the subcultured whole callus tissues became brown and necrotic after 4–6 d of culture. The autoclaved culture filtrates showed the same level of activity and their active metabolites might be highly thermostable (Verdejo-Lucas et al., 2009). Another report also confirmed the thermostable compounds of D-arabitol pentaacetate, D-mannose *p*-nitroanilide and 1,2,3,4,6α-D-glucose pentaacetate in *Puccinia graminis* var. *tritici* (Prentice and Cuendet, 1954). *In vitro* selection of fungal toxins with defensive molecules could lead to resisting the plant cells (Jayasankar and Gray, 2003).

For indirect organogenesis, microshoots formed directly from the cotyledonary leaf- and immature leaf-derived toxin resistant calli in the plant regeneration medium containing 4.0 mg/L mT (meta-Topolin) + 1.5 mg/L CPPU (Vijayakumar et al., 2017) alone or in addition with various concentrations of FCF (0–50%). In the cotyledonary leaf-derived resistant calli, the microshoot induction frequency decreased from 97.3% (52.3 shoots/callus piece) to 18.3% (5.5 shoots/callus piece) in

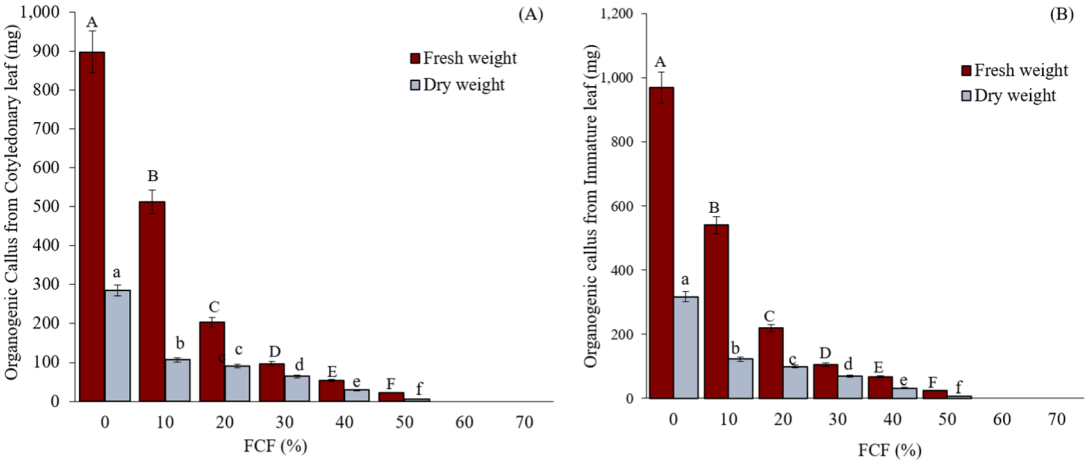

Fig. 2 Histograms showing mean production (wet or dry weight of callus, g) of fungal culture filtrate (FCF)-resistant organogenic callus from: (A) cotyledonary leaf and (B) immature leaf of safflower cv. NARI-H-15 (after 60 d), where different uppercase or lowercase letters above bars indicate significant ($p < 0.05$) difference among mean fresh weight or mean dry weight, respectively; error bar = ± SD

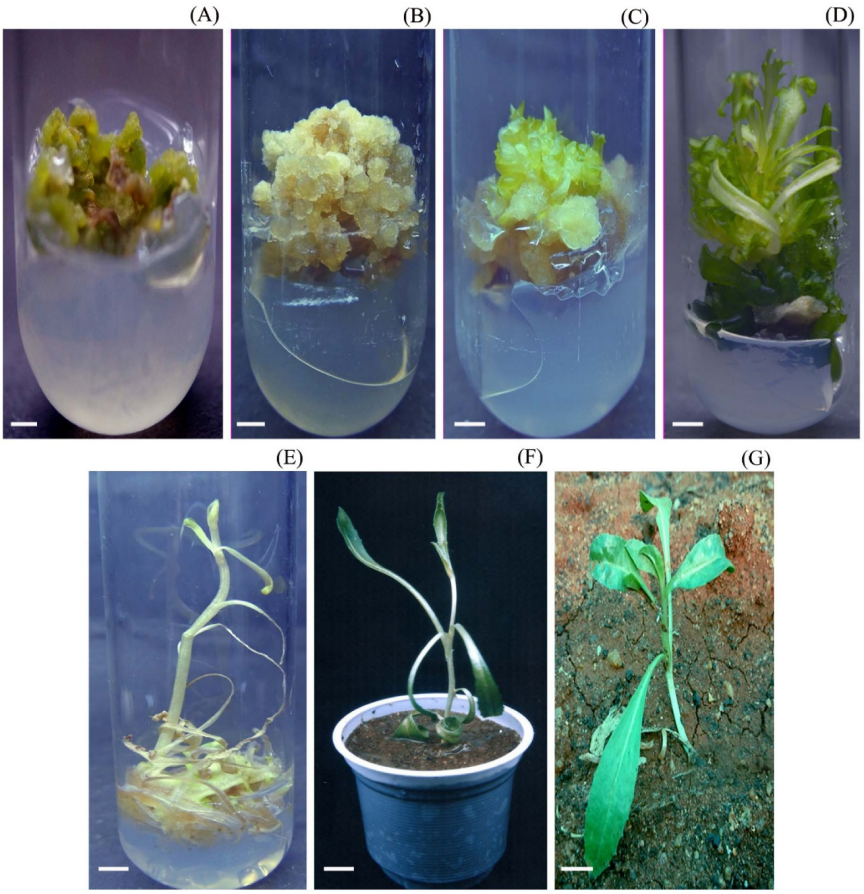

Fig. 3 Effect of fungal culture filtrate (FCF) on plant regeneration through organogenesis of safflower cv. NARI-H-15: (A) initiation of toxin resistant organogenic calli from immature leaf explants on Murashige and Skoog (MS) + 40% FCF + N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU; 1.5 mg/L) (scale bar = 5 mm); (B) proliferation of FCF-resistant organogenic calli from immature leaf explants after 60 d (scale bar = 5 mm; (C, D) initiation and proliferation of resistant shoots from immature leaf derived organogenic calli on MS + 40% FCF + 4.0 mg/L meta-Topolin (mT) + 1.5 mg/L CPPU after 55 d, where scale bar = 5 mm; (E) rooting of toxin resistant shoots on 1/4 strength MS + 40% FCF + NAA (1.5 mg/L) + 0.1 mg/L mT after 30 d (scale bar = 5 mm; (F) survival of FCF-tolerant plants on plastic cups containing sterile soil mixture (scale bar = 10 mm; (G) field survival of *Puccinia carthami*-tolerant plants (scale bar = 40 mm)

the 0–40% FCF-treatments after 55 d. for the immature leaf-derived toxin resistant calli, the plant regeneration percentages decreased from 100% (57 shoots/callus piece) to 22.8% (8 shoots/callus piece) in the 0–40% FCF-treated cultures (Table 1; Figs. 3C and D). At 50% FCF selection, a reduced frequency of shoot development without rooting was reported from cotyledonary leaf- and immature leaf-derived organogenic calli. However, the elongated healthy shoots from cotyledonary leaf-derived organogenic calli placed on rooting medium containing 0–40% FCF had decrease in the rooting frequency from 81.5% (4.1 rootlets/plantlets) to 14% (1.2 rootlets/plantlets). The isolated microshoots from immature leaf calli cultured on medium with 0–40% FCF had a decreased rooting frequency from 84.7% (4.9 rootlets/plantlets) to 16.6% (1.6 rootlets/plantlets), as shown in Table 1 and Fig. 3E. Thus, 40% FCF was a better concentration for toxin-insensitive organogenic calli production and plant regeneration. In the 40% FCF treatment experiment, 10.2 plantlets developed into complete plants that were FCF-tolerant. However, about 35% of plants adapted to pots containing a soil mixture while being maintained under greenhouse conditions (Figs. 3F and G). Similar results were reported for *Arachis hypogaea* (Venkatachalam and Jayabalan, 1996) and safflower (Vijayakumar et al., 2008b).

Effect of fungal culture filtrate on plant regeneration via somatic embryogenesis

The FCF selected medium influenced toxin-insensitive pro-embryogenic callus formation from cotyledonary leaf and immature leaf explants. The frequency of resistance and growth of the embryogenic calli were based on the concentration of FCF (0–70%) along with the optimum level of mT (1.5 mg/L) + picloram (3.0 mg/L) + CPPU (1.0 mg/L). The frequency of toxin-resistant embryogenic callus induction from cotyledonary leaf explants gradually decreased from 99% (711 mg fresh weight) to 12.5% (53 mg fresh weight) in the 0–40% FCF treatment medium after 60 d of culture. In the immature leaf explants culture, the toxin-resistant embryogenic callus induction frequency greatly decreased from 94.8% (680 mg fresh weight) to 10.3% (47 mg fresh weight) for the 0–40% FCF selection medium. Therefore, 40% of FCF was a better concentration for the regeneration of toxin-resistant embryogenic calli from cotyledon- than immature-leaf explants. Little callus growth with cell death was noticed with the 50% FCF-treated culture. FCF at 50% produced a small amount of toxin-resistant calli formation, followed by programmed cell death for both cotyledonary leaf- and immature leaf-explants culture (Figs. 4A and B; Figs. 5A and B). Explant survival was almost completely prevented at 60–70% FCF selection for all culture conditions.

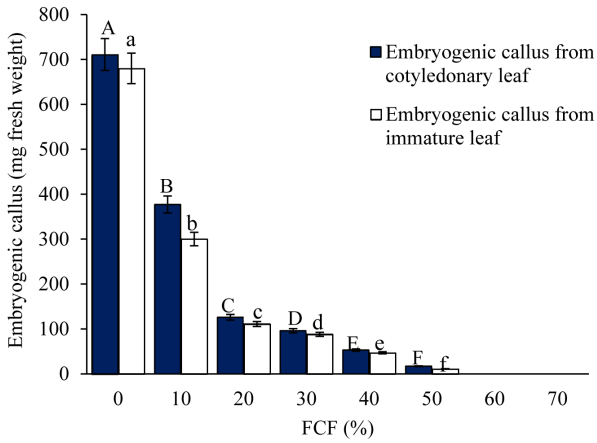

Fig. 4 Effect of *Puccinia carthami* fungal culture filtrate (FCF) on resistant embryogenic calli induction from cotyledonary leaf and immature leaf explants of safflower cv. NARI-H-15 after 60 d (expressed by callus fresh weight), where different uppercase or lowercase letters above bars for the same explant type indicate significant ($p < 0.05$) differences and error bar = ± SD

Table 1 Effect of *Puccinia carthami* fungal culture filtrates (FCF) on organogenesis from cotyledonary leaf- and immature leaf-derived callus of safflower cv. NARI-H-15

FCF concentration (%)	Cotyledon				Immature leaf			
	Mean number of callus explants	Number of Microshoots	Organogenesis (%)	Rooting (%)	Mean number of callus explants	Number of microshoots	Organogenesis (%)	Rooting (%)
0	38.9±3.8^{a}	52.3±2.1^{a}	97.3	81.5 (4.1)	40.0±0.5^{a}	57.0±2.0^{a}	100	84.7 (4.9)
10	29.0±2.0^{b}	28.7±1.8^{b}	72.5	47.0 (3.5)	35.0±1.0^{ab}	31.3±1.5^{ab}	87.5	50.0 (3.8)
20	21.7±2.2^{bc}	19.0±2.2^{c}	54.3	31.0 (2.3)	22.0±2.0^{b}	24.1±1.6^{b}	55.0	33.0 (2.9)
30	12.5±1.5^{c}	11.1±2.2^{cd}	31.3	19.0 (1.9)	13.0±2.3^{bc}	18.5±2.2^{bc}	32.5	21.5 (2.0)
40	7.3±2.0^{cd}	5.5±1.4^{d}	18.3	14.0 (1.2)	9.1±1.0^{c}	8.0±3.0^{c}	22.8	16.6 (1.6)
50	3.0±1.7^{d}	0.8±2.0^{e}	7.5	0	3.8±3.2^{cd}	1.2±2.0^{d}	9.5	0

Mean±SD within a column superscripted by different superscript letters are significantly ($p < 0.05$) different

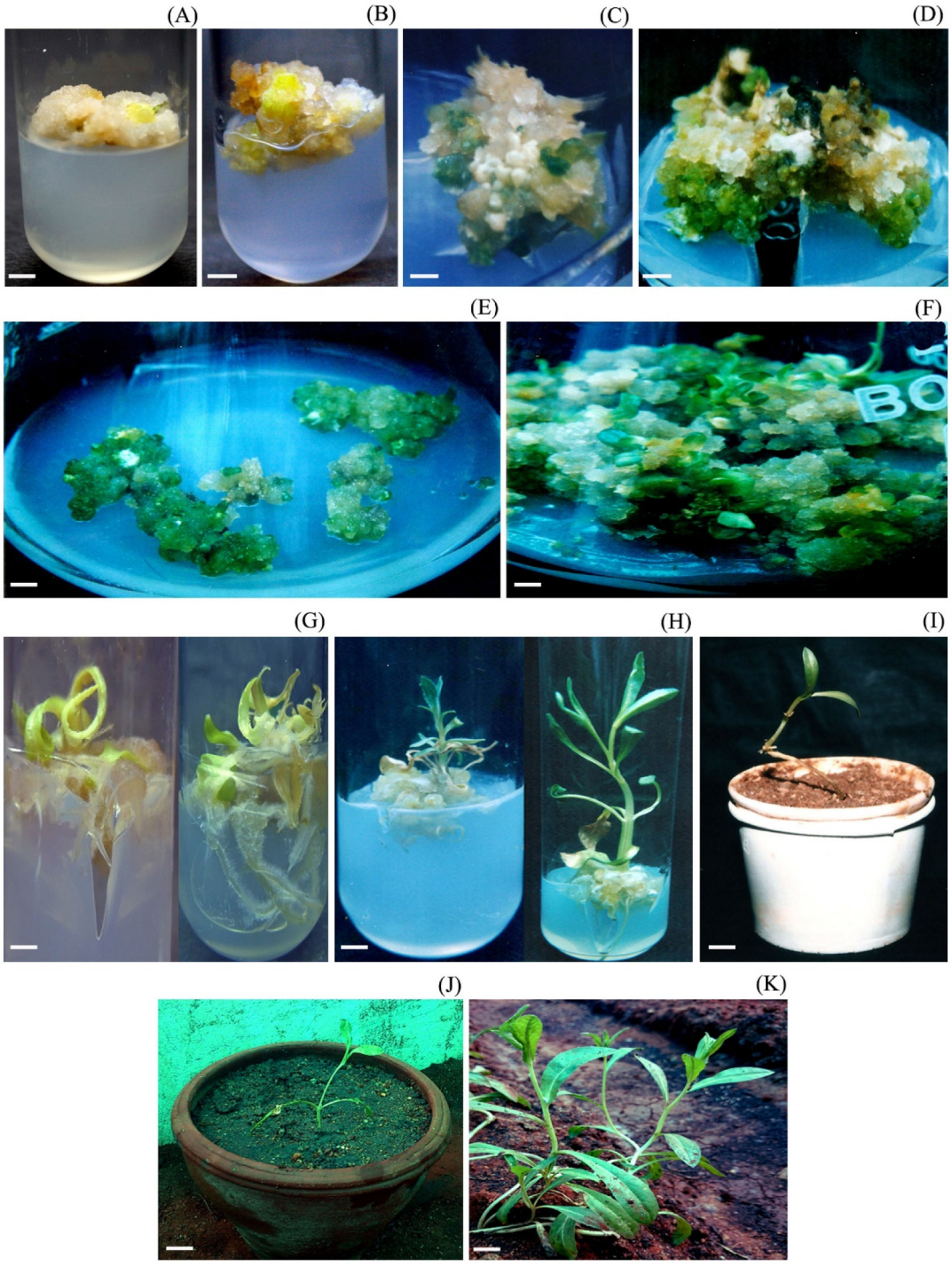

Fig. 5 Effect of fungal culture filtrate (FCF) on plant regeneration through somatic embryogenesis of safflower cv. NARI-H-15: (A, B) embryogenic calli from cotyledons on Murashige and Skoog (MS) + 40% FCF + 1.5 mg/L meta-Topolin (mT) + picloram (3.0 mg/L) + N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU; 1.0 mg/L) after 60 d (scale bar = 5 mm); (C, D, E, F) toxin-resistant embryogenic calli on MS medium + 40% FCF + polyamines (20 µM putrescine + 15 µM spermidine) and mT (4.0 mg/L) + picloram (1.0 mg/L) + CPPU (0.3 mg/L) after 45 d (scale bar = 20 mm); (G) complete embryo germination with shoot and root poles on 1/4 MS medium + 40% FCF, 1.0% sucrose, 0.8% agar, myoinositol (50 mg/L), thiamine HCl (3.0 mg/L) medium fortified with 0.3 mg/L gibberellic acid (GA3) and 0.1 mg/L mT after 7 d (scale bar = 4 mm); (H) rooting of cotyledonary stage embryos on rooting medium with 40% FCF + 1-naphthylacetic acid (NAA; 1.5 mg/L) + mT (0.1 mg/L) after 30 d (scale bar = 5 mm); (I, J) FCF-tolerant plants on plastic cups and earthen pots containing sterile soil mixture (scale bar = 10 mm); (K) the *P. carthami*-tolerant plants in greenhouse (scale bar = 45 mm)

Based on the above experiment, the recovered toxin-insensitive embryogenic calli were utilized for somatic embryogenesis using solid and suspension culture. The frequency of embryo induction was gradually decreased in the FCF (0–50%) selective medium fortified with the optimum level of polyamines (20 µM putrescine + 15 µM spermidine) mT (4.0 mg/L) + picloram (1.0 mg/L) + CPPU (0.3 mg/L). Toxin-resistant embryogenic calli were subcultured on the liquid medium for induction and proliferation of more somatic embryos. The sieved embryos along with the embryogenic callus were transferred to solid medium to influence cyclic embryogenesis. In this case, the number of somatic embryos formed in CDEC decreased from 137 (100%) to 13 (30%) in the 0–40% FCF-treated embryo induction medium after 45 d of culture (Figs. 5C, D, E, and F). The embryo germination percentage also decreased from 65 to 17 in the 0–40% FCF-treated semi-solid medium. In ILDEC, embryo formation decreased from 124 (99.9%) to 9.6 (28.7%) in the 40% FCF culture. Here, the embryo germination percentage also decreased from 56.8 to 13 in the 40% FCF-treated culture after 5–7 d (Table 2 and Fig. 5G). Germinated cotyledon embryos with root poles or non-root poles were isolated and transferred to root induction medium containing 0–40% FCF from which the adventitious rooting percentage decreased from 75 to 17.2 in root poles and from 52 to 11 in non-root poles (Fig. 5H). 40% FCF was noted as the best concentration for the regeneration of toxin-resistant plants of safflower cv. NARI-H-15. Finally, 18.3 plantlets were regenerated into complete plants as FCF-tolerant. With FCF levels above 50%, there was no positive response in the development of resistant plants from somatic embryogenesis processes. The fully regenerated FCF-tolerant plants survived well in the plastic cups/earthen pots containing the soil mixture and maintained under greenhouse conditions (Fig. 5I, J, and K). However, there was approximately 52% survival rate of safflower cv. NARI-H-15 after 2–3 wk. FCF-tolerant plants grew well into the mature stage with inflorescence head induction. This finding was in agreement with earlier reports claiming that *in vitro* selection of fungal toxic culture filtrates was suitable for the regeneration of resistant plants against pathogenic organisms (Song et al., 1994; Ganesan and Jayabalan, 2006; Vijayakumar et al., 2008b). Noctor and Foyer (1998) noted that fungal-resistant plants survived and showed normal growth through acquiring a protective mechanism for scavenging or detoxification of toxic compounds of reactive oxygen species (ROS).

Compared with organogenesis, the greatest frequencies of plant regeneration from toxin-resistant embryogenic calli were achieved using somatic embryogenesis. The surviving potted and field plants showed normal growth as parental plants. The study of organogenesis and somatic embryogenesis indicated that the 40% FCF-treated plants from somatic embryogenesis had more systemic resistance against rust disease caused by *P. carthami* fungi. *In vitro* selection of pathotoxin resulted in the regeneration of plants with greater resistance compared to untreated control regenerants. These pathotoxins can be a special defensive chemical weapon to improve plant resistance. In a similar fashion, the generation of disease resistance through the selection of pathotoxins was reported in alfalfa (Hartman et al., 1984), tomato (Scala et al., 1984), barley (Chawla and Wenzel, 1987), tobacco (Slavov et al., 1998) sunflower (Slavov and Christov, 2002), cotton (Ganesan and Jayabalan, 2006), safflower (Vijayakumar et al., 2008b) and other plants (Van den Bulk, 1991; Svábová and Lebeda, 2005). In addition, prolonged exposure of culture to FCF at higher concentrations reduced the cell growth pattern. Loss of cell growth and plant regeneration might be encountered through FCF selection at

Table 2 Effect of *Puccinia carthami* fungal culture filtrates on somatic embryogenesis from cotyledonary leaf- and immature leaf-derived embryogenic callus[a] of safflower cv. NARI-H-15

FCF concentration (%)	CDEC			ILDEC		
	Number of somatic embryos per 100 mg of embryogenic callus	Somatic embryos (%)	Germination of embryos (%)	Number of somatic embryos per 100 mg of embryogenic callus	Somatic embryos (%)	Germination of embryos (%)
0	137.0±1.5^{a}	100	65.0	124.0±3.0^{a}	99.9	56.8
10	111.0±2.0^{ab}	76.6	52.7	103.0±2.0^{ab}	71.0	49.5
20	91.7±2.3^{b}	65.0	43.0	84.0±1.8^{b}	60.2	37.0
30	72.0±1.6^{bc}	59.0	38.0	60.0±4.0^{bc}	53.5	31.3
40	13.0±2.0^{c}	30.0	17.0	9.6.0±3.8^{c}	28.7	13.0
50	2.5±3.1^{d}	11.0	0	0.9.0±2.0^{cd}	9.3	0

FCF = fungal culture filtrates; CDEC = cotyledon-derived embryogenic calli; ILDEC = immature leaf-derived embryogenic calli;
Mean±SD within a column superscripted by different superscript letters are significantly ($p < 0.05$) different

40% for long periods. Problems in plant regeneration combined with *in vitro* selection for a longer period might be associated with cytogenetic alteration (McCoy et al., 1982). Similarly, exposure of cell lines of *Gladiolus* to fusaric acid for a longer period resulted in cytogenetic alteration (Nasir and Riazuddin, 2008). In most instances, the long-term application of fungal culture filtrates may induce a long-lasting and extensive spectrum of natural disease resistance in plants grown under non-stressed field conditions, as described by Vidhyasekaran (1997). Genetic analysis may contribute to understanding the *in vitro* selection of FCF integrated with study of molecular and functional genomics to improve rust disease resistance in safflower plants.

Activity of antioxidant enzymes

The selection of FCFs in plant tissue culture medium produced a range of physiological and biochemical changes analogous to pathogenesis. Fungal culture filtrates induce defense responses and oxidative stress in plants (Saikia et al., 2006). Oxidative stress deals with the intracellular production of ROS is associated with the causes and effect of diseases. Usually, ROS production is the most primitive response of plant tissues to elicitors, pathotoxins and attack by pathogens. Similarly, Suzuki and Mittler (2006) reported that biotic stress can stimulate the production of greater amounts of ROS, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). ROS production through oxidative stress has been reported in different plant species (Unger et al., 2005; Khan et al., 2015). Other reports have identified that an antioxidative enzyme such as CAT, glutathione reductase, ascorbic peroxidase (APx), superoxide dismutase (Nunkaew et al., 2014; Gupta et al., 2019) and glutathione *S*-transferases are involved in the detoxification of ROS and other cytotoxic, genotoxic and xenobiotic compounds (Veal et al., 2002). The resistant plants might be effective in the detoxification of excessive ROS levels through activating antioxidant enzymes (Chen et al., 2015). On the other hand, pathogen-encoded molecules (elicitors) and secondary endogenous signals may activate some of the plant protectant and defense genes such as peroxidases, glutathione *S*-transferase, proteinase inhibitors, cell wall proteins, pathogenesis-related proteins, fungal cell wall degrading enzymes (such as chitinases and β-1,3-glucanases) and phytoalexin biosynthetic enzymes like phenylalanine ammonia-lyase and chalcone synthase (Hammond-Kosack and Jones, 1996). FCF stress can trigger the elicitation of various defense responsive enzymes in plant cells. In such cases, the participation of enzymes such as CAT, POD and SOD in the metabolism of ROS has been studied in relation to safflower cv. NARI-H-15 plant resistance against *P. carthami*. The SOD enzymes change the superoxide radical into hydrogen peroxide and molecular oxygen (O_2), whereas catalase and peroxidases change hydrogen peroxide into water molecules (Weydert and Cullen, 2010). Peroxidase enzymes react with H_2O_2 and produce the [Fe^{4+} = O] R' (compound I), according to Nelson et al. (1994). Peroxidase can also catalyze the reaction for the reduction of peroxide compounds into alcohol and oxygen molecules (Frank and Sosenko, 1987). Peroxidase induces plant-pathogen interactions, growth and aging of cells (Passardi et al., 2004) and somatic embryogenesis (Vranova et al., 2002). In the present study, the antioxidant enzyme activity was assessed spectrophotometrically for *in vitro*-raised control and FCF-tolerant plants. The increase or decrease in the enzyme activity of the plants coincided with a variable increment in the individual isoform expression under normal and stressful conditions. The specific CAT activity slightly decreased in 40% FCF-tolerant plants compared to control plants (Fig. 6A). The decrease in CAT activity was due to the lower affinity for H_2O_2 than peroxidase and other antioxidant enzymes (Mittler and Zilinskas, 1991). Flavonoids are inhibitors of CAT activity and might partially occurred due to the formation of hydrogen bonds between catalase and flavonoids (Krych and Gebicka, 2013). More POD and SOD activity could compensate for the low CAT activity (Vijayakumar et al., 2008b). The toxic effect of FCF resulted in more POD and SOD activity in tolerant plants than for the control. In this case, POD had high activity (34.3%) with an average of 0.37 µmol/min/mg protein in FCF-treated plants through somatic embryogenesis while the minimum activity (32.9%) was observed with an average of 0.26 µmol/min/mg protein in plants treated with FCF through organogenesis (Fig. 6B). SOD was showed more active (22.6%) with an average of 168 U/mg protein in FCF-tolerant plants from somatic embryogenesis compared to the control plants. However, the FCF-tolerant plants produced through organogenesis had 22.2% SOD activity with an average of 150 U/mg protein (Fig. 6C). The resistant response in regenerated plants is characterized by observing an increasing level of POD and SOD because of the exposure of the culture to 40% FCF. The increased activity of POD and SOD could compensate for the low CAT activity in the control and the fungal-tolerant plants of safflower cv. NARI-6 due to the response of FCF-induced oxidation damage (Vijayakumar et al., 2008b). Zhang et al. (2010) reported that leaves of male *Populus cathayana*

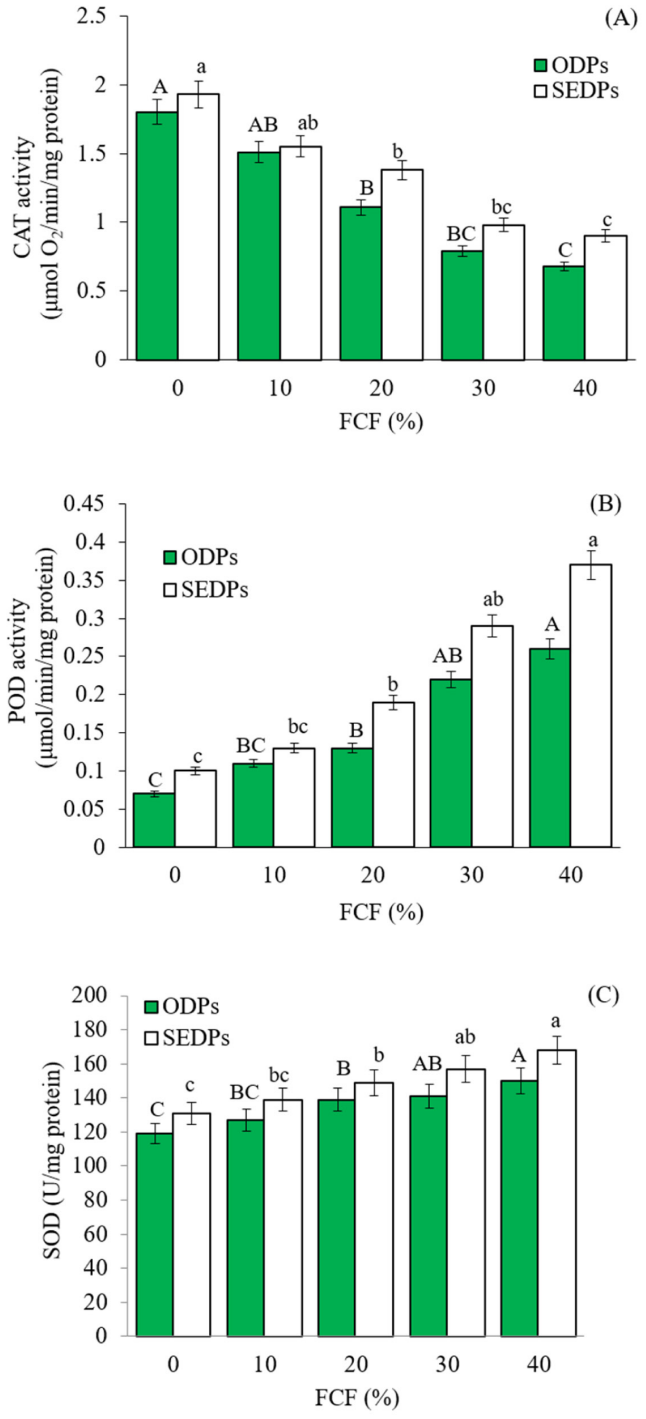

Fig. 6 Activity of antioxidant enzymes in fungal culture filtrate (FCF)-tolerant safflower cv. NARI-H-15 plants: (A) catalase (CAT); (B) peroxidase (POD); (C) superoxide dismutase (SOD), where different uppercase or lowercase letters above columns denote significant ($p < 0.05$) difference within organogenesis-derived plants (ODPs) or somatic embryogenesis-derived plants (SEDPs), respectively; error bar = ± SD

had SOD and POD activities and a lower CAT activity, while female plants had different activities due to oxidative stress induced by the rust fungus, *Melampsora larici-populina*. Zehra et al. (2017) showed that the application of signaling molecules of exogenous salicylic acid with the pathogen greatly decreasing H_2O_2 scavenging enzymes (APx and CAT) and greatly increasing SOD activity in tomato plants against *Fusarium* wilt disease. In other cases, CAT activity increased in susceptible tomato plants with fungal infection compared to healthy tomato plants (Kuzniak and Sklodowska, 2005) and was negatively correlated with other antioxidant enzymes (Fortunato et al., 2015).

Staining assay of antioxidant enzymes using native polyacrylamide gel electrophoresis

The direct involvement of enzymes in ROS scavenging activity in both *in vitro*-raised control and FCF-tolerant plants via organogenesis and somatic embryogenesis was analyzed using native PAGE. The banding intensity for these CAT, POD and SOD differed based on the enzyme activity. Thus, the banding pattern for these three enzymes did not vary between the organogenesis- and somatic embryogenesis-derived plants. CAT revealed a single thick band in the control and a reduction in banding size was observed in FCF-tolerant plants (FCF1 and FCF2) (Fig. 7A). Multiple isoforms of POD and SOD were found in the control as well as in FCF-tolerant plants. In the control plants from organogenesis and somatic embryogenesis, POD had a greater staining intensity with two (1 and 2) clear bands. However, the FCF-tolerant plants produced through organogenesis and somatic embryogenesis had a strong staining intensity with three clear bands of isoforms for POD (Fig. 7B). SOD had greater staining intensity with two isoform bands from the control plants and four isoforms bands were visualized at high intensity in native gel relative to SOD from FCF-tolerant plants from organogenesis and somatic embryogenesis, respectively (Fig. 7C). The most abundant isoforms increased in the gel stained for SOD activity, showing greater intensity. The development of the first two bands could be identical, whereas the third, faster-moving band (observed as one of the isoforms in the electropherograms revealed from POD) was FCF-specific. The second and third bands were almost identical for SOD, while the first and fast-moving fourth bands of isoforms were separated as FCF-specific in tolerant plants (FCF1 and FCF2). The present results were consistent with Badiani et al. (1990) and Naderi et al. (2014). They reported that higher SOD activity was found in a tolerant

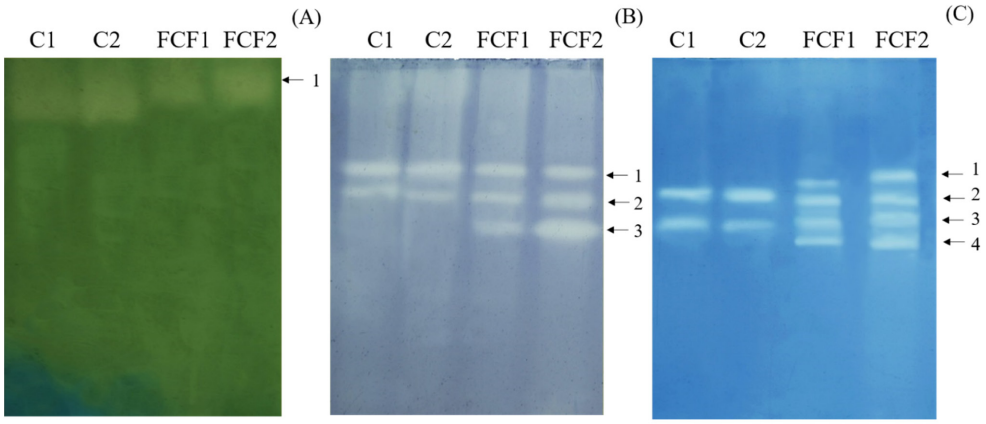

Fig. 7 Staining activity of antioxidant enzymes in 40% fungal culture filtrate-treated plants of safflower cv. NARI-H-15 using native polyacrylamide gel electrophoresis: (A) catalase; (B) peroxidase; (C) superoxide dismutase, where C1 and C2 are banding patterns of isoforms of antioxidant enzymes in control plants, FCF1 is banding pattern of isoforms of antioxidant enzymes in rust disease-tolerant plants produced from organogenesis and FCF2 is banding pattern of isoforms of antioxidant enzymes in rust disease-tolerant plants produced from somatic embryogenesis

group compared with susceptible and intermediate groups in scavenging O_2^- under severe stress conditions. The rust disease-resistant plants regenerated from cotyledonary leaf-derived embryogenic calli through somatic embryogenesis from the 40% FCF treatment had greater activity of antioxidant enzymes than from organogenesis-derived plants of safflower cv. NARI-H-15. Activation and deactivation of most of the antioxidative defense enzymes are the main characteristics in the control and fungal-tolerant plants (Anderson et al., 1995; Hernández et al., 2000; Vijayakumar et al., 2008b; Rai et al., 2011). Maintenance of greater antioxidant enzyme capacity to scavenge the toxic ROS might be associated with increased tolerance in plants (Zaefyzadeh et al., 2009; Chen et al., 2010). Furthermore, alterations in the antioxidant enzyme activity and isozyme banding patterns of CAT, POD and superoxide dismutase in the leaves of the control and rust disease-tolerant safflower cv. NARI-H-15 plants have a complex relation to the phenomenon of induced systemic resistance (ISR) by FCF. Jayasankar et al. (2000) described the combined action of several detoxifying antioxidant enzymes during *in vitro* selection induced systemic resistance in the selected lines of *Vitis vinifera*. Therefore, antioxidative enzymes can play an important role in the defense mechanism against pathogen culture filtrate or pathogenic fungus (Vijayakumar et al., 2008b; Rai et al., 2011).

Evaluation of pathogenicity

Applying a spore suspension to surviving potted and field plants is an effective method for the induction of pathogenicity. The *in vitro*-raised plants from the control and FCF-treatments were evaluated for pathogenicity. Spraying *P. carthami* fungal spores (5×10^5 spores/mL) significantly reduced the survival rate and affected the growth pattern of the control plants, whereas the symptoms of rust disease were controlled in the FCF-tolerant plants. The fungal spores of *P. carthami* sprayed on the FCF-tolerant plants had better growth with minimum numbers of reddish-brown colored rust spot lesions found after 40 d. In the present study, the symptoms of rust spot disease were evaluated in leaf and stem portions of the control and FCF (0– 40%)-tolerant plants produced via organogenesis and somatic embryogenesis of safflower cv. NARI-H-15. The surviving field FCF-treated plants through somatic embryogenesis had greater disease tolerance than the organogenic derived FCF-treated plants. The control plants were highly susceptible to reddish-brown color rust infection by *P. carthami* fungal spores on the leaves and the stem portion of plants (Fig. 8A and B). Among the seven FCF-tolerant plants tested, rust disease symptoms were observed on four plants (ODPs). An average of 0.5 and 0.8 brown irregular rust spot lesions were identified on the leaf and stem portions, respectively, of the 40% FCF-treated ODPs of safflower cv. NARI-H-15. The size of the rust spot lesion decreased from 5.2 mm to 0.3 mm on leaves, while the infected stem portions also produced rust spot lesions from 4.2 mm to 0.7 mm for the 0–40% FCF-treated ODPs. The remaining three plants showed complete disease resistance (Table 3 and Fig. 8C). In the SEDPs, among the 40% FCF-treated plants tested (14), 8 plants had averages of 0.2 and 0.5 irregular rust spot lesions on *P. carthami*-infected leaf and stem portions, respectively.

The size of the irregular rust spot lesions decreased from 3.0 mm to 0.1 mm and from 3.8 mm to 0.1 mm on infected leaf and stem portions in SEDPs, respectively, for the 0–40% FCF treatment (Table 4 and Fig. 8C). The remaining six plants had complete rust disease tolerance against *P. carthami* infection. Therefore, the 40% FCF-treated ODPs and SEDPs (R0 plants) showed 83.5 and 97%, respectively, *P. carthami* disease tolerance in safflower cv. NARI-H-15 during the maturation period in the soil. The confirmation co-determinants of pathogenicity during disease development were based on the accumulation of fungal toxins (Svábová and Lebeda, 2005) or the phenotypic response of plants to toxic culture filtrates. Entire toxic compounds of the FCFs did not show any differential activity of pathogenesis. Some toxic compounds present in the FCF might influence the low degree of pathogenesis. It was similarly observed in muskmelon cultigens (Megnegneau and Branchard, 1991). The present results showed that 40% FCF was able to inhibit rust symptoms in leaf and stem parts of *in vitro*-developed resistant plants of safflower cv. NARI-H-15 against *P. carthami* inoculation under *in vivo* conditions.

This was the first report on the production of FCF-tolerant plants from cotyledonary leaf- and immature leaf-derived calli through organogenesis and somatic embryogenesis against rust disease with increased POD and SOD enzyme activity in safflower cv. NARI-H-15. This study clearly described the synergistic effect of FCF to induce *P. carthami* disease resistance in safflower cv. NARI-H-15 and the levels of tolerance were evaluated by the estimation and analysis of defense enzyme activities. The pathogenicity assay confirmed disease resistance in the surviving field FCF-tolerant plants. Thus, this finding suggested that this may be a most promising and non-transgenic strategy for analyzing disease resistance in a number of generations to strengthen safflower germplasm improvement programs. The exploitation of this approach should provide a novel prospect for improving disease resistance against various fungal pathogens to improve the yield and stability of many safflower cultivars. In the future, ***in vitro*** selection with molecular and functional genomic approaches will provide a better choice for the development of disease-resistant plants.

Table 3 Analysis of rust disease in fungal culture filtrate-tolerant and control plants of safflower cv. NARI-H-15 produced through organogenesis, sprayed with *Puccinia carthami* spores (5×10^5 spores/mL)

FCF concentration (%)	Number of FCF tolerant plants	Organogenesis-derived plants			
		Leaves		Stem	
		Number of reddish-brown rust spot lesions/leaf (after 30 d)	Reddish-brown rust spot size (mm)	Number of reddish-brown rust spot lesions/leaf (after 30 d)	Reddish-brown rust spot size (mm)
0	27.0±2.4^{a}	6.1±3.1^{a}	5.2±1.4^{a}	3.8±2.0^{a}	4.2±1.6^{a}
10	20.0±3.0$_{ab}$	3.0±2.0^{b}	2.5±1.9^{b}	2.5±2.1^{b}	3.0±2.0^{b}
20	16.0±2.6^{b}	1.9±1.9^{bc}	1.3±2.0^{bc}	2.1±2.3^{bc}	1.8±3.4^{c}
30	13.0±1.5^{bc}	1.1±1.6^{c}	0.7±2.1^{c}	1.4±3.0^{c}	1.2±2.2^{cd}
40	7.0±2.0^{c}	0.5±1.5^{cd}	0.3±2.4^{cd}	0.8±1.6^{cd}	0.7±2.0^{d}

Mean±SD within a column superscripted by different superscript letters are significantly ($p < 0.05$) different

Table 4 Analysis of rust disease in fungal culture filtrate-tolerant and control plants of safflower cv. NARI-H-15 produced through somatic embryogenesis, sprayed with *Puccinia carthami* spores (5×10^5 spores/mL)

FCF concentration (%)	Number of FCF tolerant plants	Somatic embryogenesis-derived plants			
		Leaves		Stem	
		Number of brown rust spot lesions/leaf (after 30 d)	Brown rust spot size (mm)	Number of Brown rust spot lesions/leaf (after 30 d)	Brown rust spot size (mm)
0	68.0±2.3^{a}	4.8±3.0^{a}	3.0±1.9^{a}	3.5±3.3^{a}	3.8±1.6^{a}
10	45.0±2.0^{b}	2.6±2.1^{b}	2.1±2.9^{ab}	2.3±1.5^{b}	2.7±2.8^{b}
20	36.0±1.5^{bc}	1.5±1.8^{bc}	1.0±3.0^{b}	1.9±2.0^{bc}	1.5±2.0^{bc}
30	29.0±3.0^{c}	0.6±2.0^{c}	0.5±2.0^{bc}	1.1±3.9^{c}	1.0±4.9^{c}
40	14.0±3.9^{cd}	0.2±4.8^{cd}	0.1±1.7^{c}	0.5±4.9^{cd}	0.1±2.0^{cd}

FCF = fungal culture filtrate

Mean ± SD within a column superscripted by different superscript letters are significantly ($p < 0.05$) different.

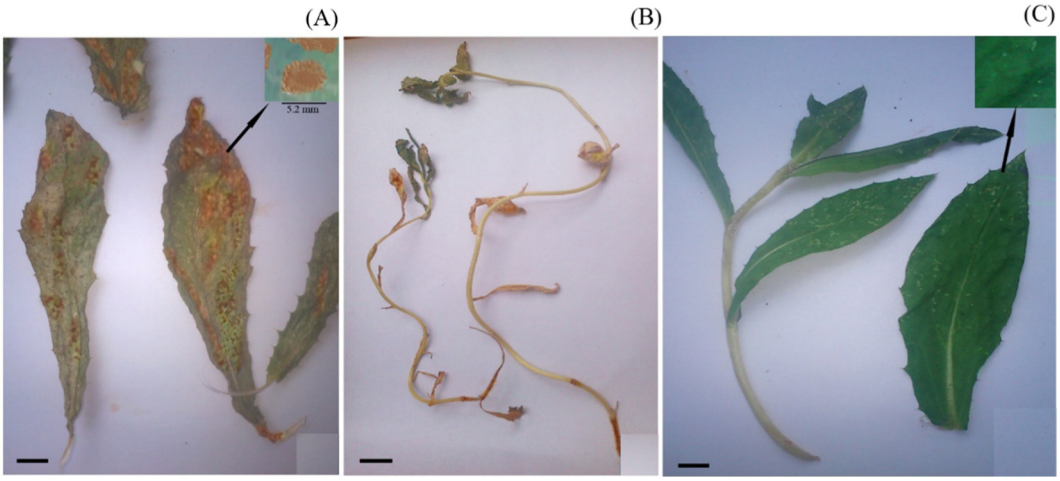

Fig. 8 Figures showing *Puccinia carthami* rust disease in safflower cv. NARI-H-15: (A) control plant leaves with number of reddish brown rust spot lesions (scale bar = 24 mm); (B) control plant stems with number of reddish brown rust spot lesions (scale bar = 40 mm); (C) fungal culture filtrate-tolerant plants without rust spot lesions (scale bar = 22 mm)

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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