IDENTIFICATION AND *IN-VITRO* ASSESSMENT OF A GLYCOPROTEIN ELICITOR ISOLATED FROM *COLLETOTRICHUM FALCATUM*: THE CAUSAL AGENT OF RED ROT IN SUGARCANE

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Abstract

The fungal pathogen *Colletotrichum falcatum,* the causal agent of red rot disease, is one of the most damaging pathogens of sugarcane (*Saccharum* spp.). The identification of elicitors that initiate defense mechanism in plants against pathogen has become an area of considerable interest in the era where scientists are aiming to reduce the use of fungicides. In this study *Colletotrichum falcatum* spores were isolated from infected stalk pieces of sugarcane and grown on Czapek Dox agar medium to obtain pure culture. Then spores were obtained from pure culture and grown in Czapek Dox liquid medium for isolation of cell wall elicitor from two week old mycelial mat of *Colletotrichum falcatum.* Crude elicitor was purified by gel filtration using Sephadex G-200 column. Twenty three fractions were collected and analyzed for the presence of sugars and proteins for further identification of elicitor molecule. Sugarcane callus was prepared from explants of red rot resistant genotype HSF-240 on agar solidified MS basal medium augmented with 3-4 mg L^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid). Suspension cultured cells were prepared after formation of white friable callus which was used for further studies. The third fraction (F3) induced the highest PAL activity (2.181 μ mol g^{-1} f. wt. h^{-1}) and phenolic contents (0.196 mg ml^{-1}) 6 h after treatment in sugarcane suspension-cultured cells of the resistant genotype, HSF-240, and was considered to have the active elicitor molecules. Moreover, this fraction itself had higher soluble proteins (0.999 mg ml^{-1}) and sugars (1.87 μg ml^{-1}) which confirmed that the elicitor molecules were glyco-protein in nature. The molecular weight of the elicitor molecule was calculated by drawing a regression plot from the regression equation (y = -66658x + 3^6) and estimated to be approximately 2133.446 kDa.

Key words: *Colletotrichum falcatum,* Glycoprotein, Elicitor, Red rot, Sugarcane.

Introduction

Sugarcane (*Saccharum* spp.) is one of the most important cash crops grown in tropical and sub-tropical regions of the world. It is the main source of commercial sugar production world-wide and accounts for two thirds of the total sugar produced (Sharma & Tamta, 2015; Khalid *et al.*, 2019). The crop is cultivated on approximately 23.6 million hectares world-wide with a gross production of 1.9 billion tones (Anon., 2018). The global sugar demand is expected to increase to 203 Mt by 2028 and this will add 32 Mt to the existing tonnage (Hossain *et al.*, 2020). In 2013-14 sugarcane was cultivated in Pakistan over 1.1725 million hectares with an overall production of approximately 67.46 million tones (Anon., 2015). Sugarcane is also a source of ethanol and bagasse production.

Among sugarcane diseases, the red rot is one of the oldest known diseases. It is of great economic importance and a serious threat to the sugar industry particularly in the tropical and sub-tropical countries (Ashwin *et al.*, 2017). The disease is caused by the fungus *Colletotrichum falcatum* Went (Perfect Stage: *Glomerella tucumanensis* (Speg.) Arx & E.Müll.) and is known to cause significant reduction in the quality and yield of susceptible sugarcane cultivars. Occurrence of the disease has been recorded in 68 sugarcane producing countries and a decrease in sugarcane yield by 5–50% was observed. It can reduce cane weight by up to 29% and loss in sugar recovery by 31% (Hossain *et al.*, 2020). Besides reducing yield attributes, red rot reduces the quality of sugarcane juice as well as quantity of

commercial cane sugar by reducing the sucrose in the cane and increasing the molasis content, thus also called the cancer of sugarcane (Sharma & Tamta, 2015). The disease is also responsible for eliminating several important sugarcane cultivars from cultivation and continues to be a problem in many countries such as USA, Bangladesh, India, Australia and Thailand (Alvi & Iqbal, 2014; Vishwanathan *et al.*, 2020).

Like other pathogens, *Colletotrichum falcatum* infects the host plant by appressoria and haustoria by penetrating inside the epidermis of host plant. The first external symptom of red rot appears mostly on the leaves. However, the pathogen infects different organs of the sugarcane thereby causing disease, and is mainly considered a disease of seed-piece and stalks (Raid, 2006). The main determinants of infection spread are sugarcane variety's susceptibility, crop management and the environmental conditions where it is being grown (Ishtiaq *et al.*, 2019).

Use of fungicides and cultivation of resistant genotypes is a common practice for management of the disease. However, new strains of the pathogen frequently emerge that inherit immunity against the fungicides. Moreover, most of the newly developed resistant sugarcane cultivars are infected by the pathogen even before they gain popularity. The reason for this is the highly variable and genetically changing nature of the pathogen which results in continuously developing new and more virulent races (Viswanathan, 2017; Viswanathan & Selvakumar, 2020). So, even if a disease resistant variety is released for cultivation, within the next 8-10 years it falls prey to the disease (Bharti *et al.*, 2014).

Host plants have the ability to perceive pathogen associated molecules as a first level of defense. This results in plant resistance as a result of effective defense response triggered by the recognition of specific pathogen molecules. In higher plants these signaling molecules may be elicitors specific to a particular strain or other phytopathogen origin chemical derivatives (Ors *et al.*, 2019). So far elicitors have been isolated from various organisms, including bacteria, viruses, oomycete and fungi (Chen *et al.*, 2014). The structure and chemical nature of elicitors is highly conserved and may consist of a variety of different molecules such as peptides, polysaccharides, glycoproteins, glycosphignolipids etc. Moreover, these elicitor molecules induce and activate defense responses in host plants (Pettongkhao & Churngchow, 2019).

Reducing the use of pesticides has become a priority in many countries with the aim of environmental sustainability and health protection. In this regards, the use of elicitors is a promising alternative to fungicides. Elicitor molecules stimulate the immune response and boost the defense state in the host plants. They activate secondary metabolic pathways and mobilize signaling molecules involved in early signaling response to pathogens (Ben Salah, 2018). Elicitor molecules may induce oxidative bursts trapping the pathogens in dead cells. They also induce changes in cell wall composition making them impenetrable to the pathogen as well as synthesis of antimicrobial compounds, ultimately inducing resistance in the host plant. Thus, elicitors are a potential solution to reducing the use of fungicides (Naveen *et al.*, 2013; Le Mire *et al.*, 2019).

Various biochemical compounds and enzymes can be used to determine the induction of pathogen resistance in host cells. Phenylalanine ammonia lyase is one of the most important plant enzymes of the phenylpropanoid metabolic pathway. It is known to induce defense related changes in the host tissue. In some previous studies, the increased activity of PAL enzyme has been shown to have a positive correlation with red-rot resistance of sugarcane (Vishwanathan, 2012; Sharma & Tamta, 2015). Other molecules produced as a result of pathogen attack include a variety of phenolic compounds (Tahsili *et al.*, 2014).

The current study was conducted to identify the chemical nature of *Colletotrichum falcatum* associated elicitor molecule isolated from cell wall of red rot fungus. In addition, the elicitor molecule was evaluated for elicitation of PAL enzyme activity and accumulation of phenolics in sugarcane cell suspension.

Materials and Methods

Growth and preservation of fungal (*Colletotrichum falcatum*) colony: Infected cane pieces (1-1.5 cm) were obtained from the Shakarganj Sugarcane Research Institute (SSRI), Jhang and placed on autoclaved agar (2%) water medium in Petri plates. The fungal colonies were allowed to grow for 7-8 days in dark at $25°C$ in an incubator and identified under microscope. Pure fungal colonies were obtained on Czapek Dox agar Medium (Czapek, 1902-1903; Dox, 1910) and verified by Koch's postulates (1893) (Table 1).

Table 1. Czapek Dox agar medium (for 100 ml).

Nutrient	Quantity
$NaNO_3$	0.3 g
K_2HPO_4	0.1 g
$MgSO_4$	0.05 g
KCl	0.05 g
$FeSO_4$	0.001 g
Sucrose	3 g
Agar	1.5 g
pH	6.4 to 6.5

*To avoid bacterial contamination, an antibiotic chloromycetin as 250 mg tablet / 500 mL was added just before pouring in Petri plates

Collection of spores in water suspension: For the extraction of fungal spores, fresh purified fungal colonies were used. After sufficient growth of fungus, autoclaved distilled water was poured into Petri-plates containing pure colonies and swirled gently. The distilled water containing the spore suspension was then collected in a sterilized test tube.

Spore count using haemocytometer: Fungal spores were identified and number of viable spores mL^{-1} of suspension were counted using a haemocytometer under a microscope (Model Zeiss). The total number of spores per mL was calculated by the following formula:

Number of spores in four cubes = 4 x 16 x 10^4 spores mL^{-1}

Preparation of cell wall elicitor of fungus: The fungal cell wall elicitor was prepared following the method of Albersheim and Anderson-Prouty (1975). Monoconidial culture of the fungus was multiplied on Czapek Dox liquid medium pH 6.4 at 25°C in 100 mL glass jars. One ml fungal spore suspension containing 3.072 x 10^6 spores/ml of *Colletotrichum falcatum* was added to each jar. The jars were incubated at 25°C in the dark in a refrigerated incubator keeping the surface flat to provide maximum surface area for cultures. A control culture medium without fungal suspension was also prepared to serve as blank.

Extraction of mycelial cell wall: Fungal mycelial mats were harvested from two week old liquid cultures using cheese cloth. Mycelia were homogenized using 5 ml of double distilled water per g wet weight. The filtrate was collected using a coarse filter paper (or coarse sintered glass funnel) and the residues obtained on the filter were saved. The residues were homogenized three more times in water and once in chloroform: methanol (1:1) and finally in acetone. The preparation was air-dried. This was referred to as mycelial cell wall fraction.

Preparation of crude extract of elicitor: One g of above extracted mycelial cell wall was suspended in 100 ml distilled water and autoclaved for 20 minutes at 120°C. The autoclaved suspension was filtered through a coarse sintered glass funnel (or coarse filter paper) and centrifuged at 9000 rpm at room temperature for 10 min. till solution was clear. The extract was concentrated to 10 mL under reduced pressure (at $-40°C$ and 0.003 mbar) in a freeze drier (Model Christ, Alpha 1-4 LD).

Partial purification and determination of molecular weight of elicitor: For determination of the molecular weight of elicitor the crude elicitor was purified by gel filtration using Sephadex G-200 column (1.5x45 cm) as described by Ramesh-Sunder *et al*., (2002). The void volume of the column was determined from the elution pattern of the tracking dyes Blue Dextran 2000 (D5751, Sigma; Mol. Wt. 2000 kDa) and yellow peridoxal 5′ phosphate (P9255, Sigma; Mol. Wt. 247.14 Da). One ml of crude extract was applied to the column and 3 ml fractions were collected by passing degassed distilled water through the column. Twenty three fractions were separated after disposing 5 ml of void volume which was supposed to have no elicitor molecules. The column was run with crude elicitor extract for five times and fractions corresponding to carbohydrate and protein peaks were pooled to make a volume of 15 ml in each fraction (Ramesh-Sunder *et al*., 2002). The pooled fractions containing the elicitor were freeze dried under reduced pressure (at -40°C and 0.003 mbar) in a freeze drier (Model Christ, Alpha 1-4 LD) and the final concentration of partially purified extract of elicitor was made 0.1 g per 100 ml of distilled water which was further used in biochemical assay. The molecular weight of the elicitor was computed from a standard plot developed from eluate volume against molecular weights of both tracking dyes as described by Whitaker, (1963), Andrews (1964) and Feliss & Martinez-Carrion (1970).

Biochemical analysis of elicitor: The collected fractions and a control were analyzed for hexose by anthrone method (Dische, 1962) and protein content by modified Lowry Assay method (Bensodoun & Weinstein, 1976).

Callus induction and maintenance: The induction and maintenance of sugarcane callus was carried out on agar solidified MS basal medium (Murashige & Skoog, 1962) supplemented with 3-4 mg L^{-1} 2, 4-D (2,4-dichlorophenoxy acetic acid) (Table 2). The shoot apical meristems (explants) were sterilized with ethanol (70%) and washed twice with sterilized distilled water. The 1-2 mm thick explant disks were cut and placed on sterilized callus induction medium in glass tubes and incubated in a growth room at 26±1$^°$C under dark conditions for two weeks for callus induction. After two weeks, the culture tubes were transferred to 16/8 h day/night photoperiod for further growth. The conditions of growth room were: PAR 1200 μ mol m^{-2} s^{-1}; RH 60%, temperature 27±1. After 6 weeks of growth in the induction medium, the callus was sub-cultured on maintenance medium (same composition to that of callus induction medium) under aseptic conditions.

Table 2. Stock solutions used for callus induction (Murashige & Skoog, 1962).

Salt	Amount used for preparation of stock solution L^{-1}	Volume of stock solution to be used for 1 L working solution
Micronutrients (20x)		
NH$_4$NO$_3$	33 g	
KNO$_3$	38 g	
CaCl$_2$.2H$_2$O	8.8 g	50 ml
MgSO$_4$.7H$_2$O	7.4 g	
KH$_2$PO$_4$	3.4 g	
Micronutrients (100x)		
MnSO$_4$	2.23 g	
ZnSO$_4$	0.86 g	
H$_3$BO$_4$	0.62 g	
KI	0.083 g	10 ml
NaMoO$_4$	0.025 g	
CoCl$_2$	0.0025 g	
CuSO$_4$	0.0025 g	
Fe EDTA (200x)		
FeSO$_4$	0.557 g	5 ml
Na EDTA	o.745 g	
Vitamins (100x)		
Glycine	0.2 g	
Nicotinic acid	0.05 g	10 ml
Pyridoxine HCl	0.05 g	
Thiamine HCl	0.01 g	
Carbon source		
Sucrose	30 g (3%)	3 %
Solidifying agent		
Agar	0.7 or 0.8%	0.7 or 0.8%
i) Callogenesis		
2,4-D (Dichlorophenoxy acetic acid)	1 mg	3.5 ml

The pH of the working solution was adjusted to 5.74 using 1N NaOH

Preparation of suspension cultured cells of sugarcane: Hundred ml Erlenmeyer flasks were washed and sterilized. Liquid MS medium (without agar) was prepared and pH was adjusted to 5.74 using 0.1 M NaOH (Table 2). Twenty ml of liquid MS medium was poured into each flask and autoclaved for 15 min at standard recommended temperature and pressure. The flasks were allowed to stand in tissue culture room for three days to check for contamination. Approximately 0.5 g friable and whitish callus was added to each flask which was then placed on orbital shaker at 28°C and 100-120 rpm for five days. The flasks were checked for contamination.

Application of fungal elicitor to cell suspension: Solutions for determination of cell wall elicitor from collected fractions were prepared with a concentration of 0.1 g in 100 ml autoclaved deionized double distilled water. In each flask containing sugarcane suspension cultured cells, 100 µl cell wall elicitor was added and flasks were again placed in an orbital shaker. Suspension-cultured cells were collected 6 h after treatment with elicitor for biochemical analysis by centrifugation at 1000xg at 4°C for 5 min.

Biochemical analysis of sugarcane suspension cultured cells: Further biochemical tests were performed after applying the elicitor fractions to suspension culture of the resistant sugarcane genotype HSF-240 and changes in total phenols and phenylalanine ammonia lyase (PAL) activity was determined 6 h after treatment with each elicitor fraction. The fractions causing a significant change in both these parameters were considered to contain elicitor molecules and used for the determination of molecular weight.

Total phenols: Total phenols were determined following the method of Folin-Ciocalteu Assay (Singleton & Rossi, 1965). A standard curve was made for working standards of tannic acid to and concentration of phenols was calculated in $mg\ g^{-1}$.

Phenylalanine ammonia lyase (PAL) EC 4.3.1.5: The activity of PAL was determined following the method of Ke & Saltveit (1986) and calculated as concentration/ amount of cinnamic acid produced per g of suspension-cultured cells.

$$A_o = \varepsilon \times c \times L$$

where, ε = Extinction coefficient (16596 $L\ mol^{-1}\ cm^{-1}$ for cinnamic acid); A = Absorbance; L = length of cuvette (1 cm); c = concentration of cinnamic acid calculated as (C = $A_o / (\varepsilon \times l)$ = $mol\ L^{-1}$ = $mM\ ml^{-1}$)

PAL activity was then calculated as:

Rate of PAL activity = $(\Delta OD/\varepsilon)$ x dilution factor x 2 x2 ($mM\ g^{-1}$ cells h^{-1}).

Statistical analysis

The data obtained for biochemical analysis was analyzed statistically by two-factor factorial Analysis of Variance (ANOVA) technique using a CoStat Computation Package (Cohort, CA, USA). The LSD values (5%) were calculated and used to test significance of means values.

Results

Soluble sugars: The concentration of soluble sugars varied significantly in all the elicitor fractions collected by gel filtration (Fig. 1). The highest amounts of soluble sugars were collected in fractions F23, F2 and F22 (3.58, 3.32 and 3.02 $\mu g\ ml^{-1}$, respectively). This was followed by average amounts of soluble sugars in fractions F9, F3, F21 and F4 (1.91, 1.87, 1.72 and 1.53 $\mu g\ ml^{-1}$), respectively. No soluble sugars were detected in fractions F5, F8, F14, F15, F16, F17 and F18.

Soluble proteins: Variations were also seen in the soluble protein content in all 23 fractions collected at 3 min. intervals (Fig. 1). The maximum soluble protein content were observed in the 22nd fraction collected after 66 min (1.545 $mg\ ml^{-1}$). Although the fractions F 17 and F 18 also showed considerable soluble proteins (0.966 and 1.023, respectively), however, they lacked any soluble sugars. On the other hand F3 comprised 0.999 $mg\ ml^{-1}$ soluble proteins and was considered a likely candidate. The least amount of soluble proteins was observed in the 10th fraction collected after 30 min. (0.02 $mg\ ml^{-1}$) (Fig. 1).

Post-elicitor response of callus: Six hours after application of each elicitor fraction to the suspension culture of the red-rot resistant genotype HSF-240, changes in total phenols and PAL activity were recorded:

Changes in concentration of phenols: Changes in phenol content due to post-elicitor response are depicted in (Fig. 2). The amount of total phenols in control suspension-cultured cells of HSF-240 (with no elicitor treatment) was 0.133 $mg\ ml^{-1}$. The highest amount of phenols were recorded for the third fraction (0.196 $mg\ ml^{-1}$) followed by F19 (0.170 $mg\ ml^{-1}$). Only two other fractions i.e. F15 and F18 gave total phenols greater than control calli (0.138 and 0.136 $mg\ ml^{-1}$, respectively). All other fractions had no positive effect on the accumulation of total phenols in calli of sugarcane cultivar HSF-240 and the amount of total phenols ranged from 0.007 $mg\ ml^{-1}$ in F10 to 0.129 $mg\ ml^{-1}$ in F16 (Fig. 2).

Changes in PAL activity: Post-elicitor PAL activity is also shown in (Fig. 2). The PAL activity in the control suspension-cultured cells was 1.217 $\mu\ mol\ g^{-1}$ f. wt. h^{-1}. After 6 hour of elicitor treatment an exceptionally high PAL activity was recorded in F3 which was 2.181 $\mu\ mol\ g^{-1}$ f. wt. h^{-1} followed by F23 (1.567 $\mu\ mol\ g^{-1}$ f. wt. h^{-1}). The fractions F2, F15, F21 and F22 also showed minor increase in PAL activity over control (1.374, 1.314, 1.289 and 1.350 $\mu\ mol\ g^{-1}$ f. wt. h^{-1}, respectively. The F8 showed the same activity as of control (1.217 $\mu\ mol\ g^{-1}$ f. wt. h^{-1}). PAL activity showed a decrease with all other fractions ranging from 1.048 $\mu\ mol\ g^{-1}$ f. wt. h^{-1} in F6 to the minimum PAL activity in F1 (0.277 $\mu\ mol\ g^{-1}$ f. wt. h^{-1}) (Fig. 2).

All fractions which induced lower PAL activity in sugarcane calli 6 h after treatment (F1, F4, F5, F6, F7, F9, F10, F11, F12, F13, F14, F16, F17, F18, F19, F20) and lower amounts of total phenols (F1, F2, F4, F5, F6, F7, F8, F9, F10, F11, F12, F13, F20, F21, F22, F23) than control

values were not considered to have the elicitor molecules (Figs. 1 & 2). Of the remaining two fractions F3 and F15, the PAL activity and total phenols in F15 were almost similar to control values. However, an exceptional increase was recorded for both PAL activity and total phenolic content 6 h after treatment by the third fraction (F3) in the callus of the resistant sugarcane genotype HSF-240. On the basis of these findings, the third fraction (F3) was considered to have the active elicitor molecules. At the same time this fraction had an adequate quantity of soluble proteins and sugars which indicated that the elicitor molecules might be glycoprotein in nature (Fig. 1).

Molecular weight of elicitor: The molecular weight of elicitor was determined by using two tracking dyes Blue Dextran 2000 (Mol. Wt. 2000 kDa) and yellow peridoxal 5′ phosphate (Mol. Wt. 247.14 Da). Since the fraction (F3) containing the active elicitor molecules was eluted within the void volume for Blue Dextran, the molecular weight of the elicitor was concluded to be greater than 2000 kDa. A regression plot was drawn and from the regression equation (y = -66658x + 3^6), the molecular weight of this glycol-proteinious elicitor was estimated to be approximately 2133.446 kDa (Fig. 3).
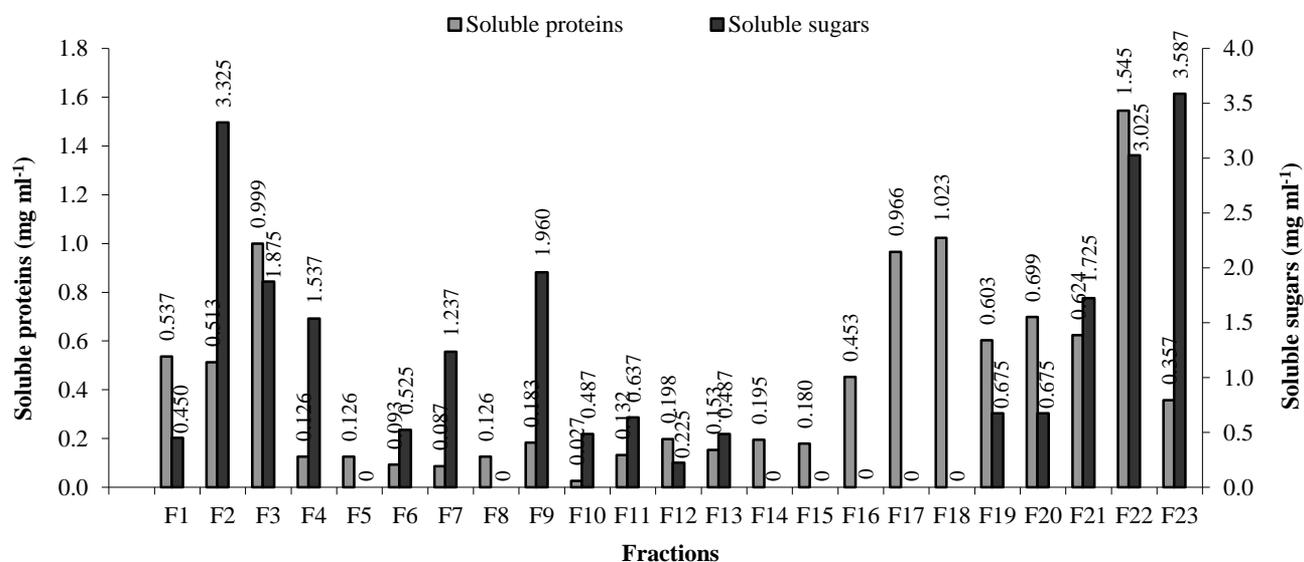
Fig. 1. Concentration of soluble sugars and proteins in various fractions of the elicitor obtained from *Colletotrichum falcatum* mycelial mat and partially purified by gel filtration using a Sephadex G-200 column. The y-axis on the left shows the concentration of soluble proteins (mg ml^{-1}), whereas the right y-axis represents the total soluble sugars (μg ml^{-1}). F 1 to F 23 on the x-axis represents the 23 fractions collected by gel filtration. The concentrations of soluble proteins and soluble sugars are written above their respective bars.
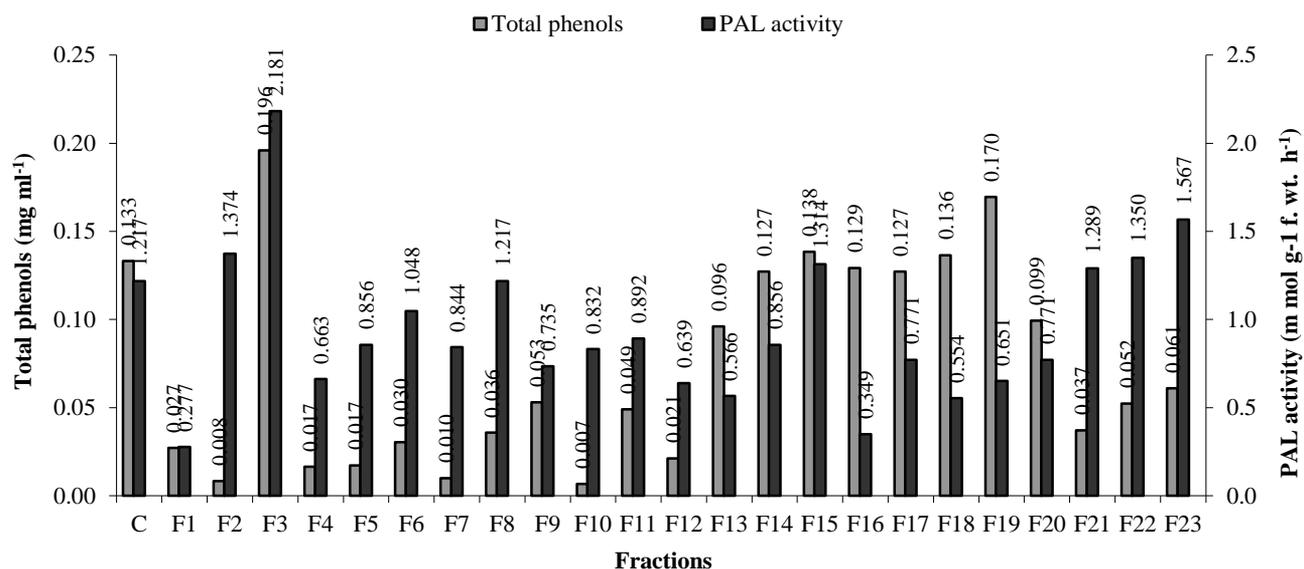
Fig. 2. Phenylalanine ammonia lyase (PAL) activity and total phenols in the suspension-cultured cells of the resistant sugarcane cultivar HSF-240 6 h after treatment with elicitor fractions of *Colletotrichum falcatum* partially purified by gel filtration using a Sephadex G-200 column. The y-axis on the left shows the concentration of total phenols (mg ml^{-1}), whereas the right y-axis represents the PAL activity recorded in suspension-cultured cells of HSF-240 (μ mol g^{-1} f. wt. h^{-1}). F 1 to F 23 on the x-axis represents the 23 fractions collected by gel filtration and C is the control sample. The concentration of total phenols and PAL activity is written above the respective bar.
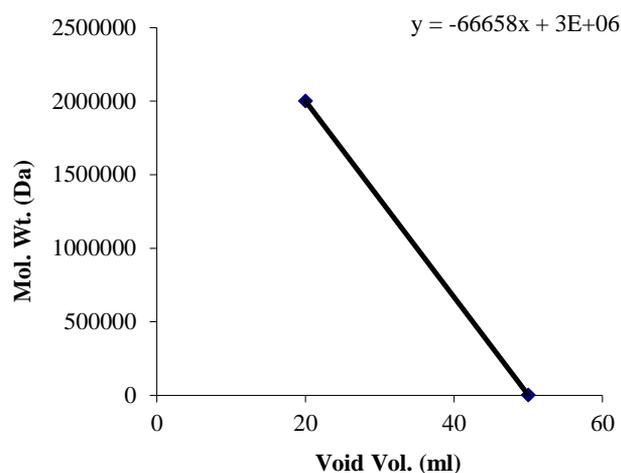
Fig. 3. A regression plot showing a trend line fitted on known molecular weights of the two tracking dyes Blue Dextran 2000 (Mol. Wt. 2000 kDa) and yellow peridoxal 5′ phosphate (Mol. Wt. 247.14 Da).

Discussion

Induction of disease resistance in plants by elicitor molecules is linked to increase in enzyme activity like that of the metabolic pathways linked phenyl-propanoid metabolism (e.g. PAL) accompanied by elevated levels of phenolic contents. Many researchers have associated increased pathogen resistance with increase in phenolic content in plants and have demonstrated that treatment of susceptible plants with precursors of phenolic compounds such as quinic acid and phenylalanine increased their phenolic content and their resistance to various pathogens. Moreover, many phenolic compounds are known to possess antimicrobial properties, function as precursors to structural polymers such as lignin, or serve as signal molecules to activate plant defense genes (Javaraj *et al.*, 2010; Vishwanathan, 2012). Thus, changes in these biochemical attributes could be directly linked with elicitation of defense responses against pathogens like *Colletotrichum falcatum.*

In the current experiment fungal elicitor was isolated from the mycellial cell wall of *Colletotrichum falcatum* and partially purified and fractioned by gel filtration. Twenty three fractions were collected and then analyzed for soluble sugar and protein contents. Biochemical tests were performed by each fraction by applying it to suspension culture of the resistant genotype HSF-240 and changes in total phenols and phenylalanine ammonia lyase (PAL) activity was determined 6 h after treatment with each elicitor fraction. A fraction giving a well-coordinated activity of PAL with phenols in suspension-cultured cells of the resistant genotype HSF-240, 6 h after treatment with elicitor and at the same time itself possessing sufficient sugars and protein content was supposed to have the elicitor molecule (Ramesh-Sundar *et al.*, 2008).

From the results of the study on fungal elicitor fractions, it was determined that the third fraction (F3) was considered to have the active elicitor molecules since the highest PAL activity (2.181 μ mol g^{-1} f. wt. h^{-1}) and

phenolic contents (0.196 mg ml^{-1}) were induced in sugarcane suspension-cultured cells of the resistant genotype, HSF-240, 6 h after treatment by this fraction. Moreover, this fraction itself had reasonable soluble proteins (0.999 mg ml^{-1}) and sugars (1.87 μg ml^{-1}) which confirmed that the elicitor molecules were glyco-protein in nature (Ramesh-Sundar *et al.*, 2002). These results indicated that the fungal elicitor induced plant defense response by triggering the activity of PAL and thereby enhanced synthesis and accumulation of phenols as also indicated in previous studies (Li *et al.*, 2011; Zhang *et al.*, 2011; Vishwanathan, 2012; Tahsili *et al.*, 2014; Sharma & Tamta, 2015).

The 22nd fraction of the elicitor contained the highest protein (1.545 mg ml^{-1}) content, at the same time, this fraction also contained exceptionally high sugar content (3.025 μg ml^{-1}). Therefore, this fraction was supposed to contain small polysaccharides, polypeptides and/or glycoprotein molecules that got eluted from the column at the end (Pessoni *et al.*, 2005). The 14th to 18th fractions consisted only of proteins and no soluble sugars were recorded. They had no effect on phenol accumulation in sugarcane suspension-cultured cells. Moreover, they stimulated significantly lower PAL activity. This indicated that these fractions contained only medium sized structural proteins of the fungal cell wall (Fontaine *et al.*, 1997). Although, the 23rd fraction showed the highest soluble sugars among all fractions (3.587 μg ml^{-1}) followed by the 2nd fraction (3.325 μg ml^{-1}), both these fractions had significantly low protein contents i.e. 0.357 and 0.513 mg ml^{-1}, respectively. In addition, they also induced little PAL activity and the phenolic contents were significantly lower in cells treated with these fractions. Therefore, they were believed not to have the elicitor molecules but sugars and polypeptides of various sizes that had the ability to trigger some PAL activity. It could be that the 23rd fraction had small sugar molecules and polypeptides while the 2nd fraction contained large polysaccharides such as α and β (1-3)-glucans complexed with chitin to form polymers making an integral component of fungal cell wall (Leal *et al.*, 1995; Kang *et al.*, 2018). All other fractions had no significant effect on enhancing the accumulation of phenols and PAL activity in sugarcane suspension-cultured cells and were assumed to consist of only structural molecules.

The molecular weight of the glycoprotein elicitor isolated from red rot fungus *Colletotrichum falcatum* from the present study was estimated to be approximately 2133.446 kDa. This is in line with other researchers who have also shown that the elicitor is a glycoprotein of high molecular weight (Ramesh-Sundar *et al.*, 2002; 2008). In other studies, the elicitors isolated from fungal pathogens *Verticillium dahliae* (*Verticillium* wilt in cotton), *Magnaporthe oryzae* (rice blast fungus), *Alternaria tenuissima* (a ubiquitous pathogen), *Phytophthora palmivora* (stem rot in durian and rubber trees) and *Phytophthora colocasiae* (leaf blight of taro) have also been reported to be glycoproteins of high molecular weight (Yang *et al.*, 2009; Mishra *et al.*, 2009; 2010; Bu *et al.*, 2014; Chen *et al.*, 2014; Pettongkhao & Churngchow, 2019).

Elicitor molecules have the potential to be used to induce systemic acquired resistance in compatible host plants. Thus, making them capable of defending against pests by inducing a variety of structural changes and synthesis of anti-microbial chemicals. Moreover, a single type of elicitor can confer resistance to a broad range of parasites. The use of environmentally safe elicitor molecules to activate desired resistance in plants may be the most effective method of achieving crop protection (Mousavi *et al.*, 2017).

Acknowledgement

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