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Role of hydrolytic enzymes in Stem Rot of Groundnut (*Arachis hypogaea* L.) caused by *Sclerotium rolfsii* Sacc.

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The present investigation was carried out in respect of activity and variability among 10 (ten) different isolates of *S. rolfsii* Sacc. under *in vitro* conditions. For *in vivo* condition only Isolate 10 was used and activity of enzymes produced by the pathogen was observed at three different stages after inoculation. *In vitro* observations revealed that highest activity of enzyme cellulase *was* produced by Isolate 6 and 8 (333.3) followed by Isolate 10 (322.5), Isolate 5 (285.0), Isolate 7 (256.4), Isolate 1 and 9 (250.0), Isolate 2 (243.9), Isolate 3 (117.6) and Isolate 4 (166.6) respectively. Similarly highest activity of enzyme pectinase was observed in Isolate 5 (50.0) followed by Isolate 3 (18.18) Results revealed that high cellulase activity and variability exists between different isolates of *S. rolfsii. In vivo* study revealed that activity of cellulase increased from Stage 1 (3 days after inoculation) to Stage II (5 days after inoculation) and there after decreased slightly at Stage III (7 days after inoculation). But in case of pectic enzymes it decreased from Stage I to Stage II and from Stage II to Stage III.

Key words: *Sclerotium rolfsii,* cellulase, pectinase, *In vitro, In vivo.*

INTRODUCTION

Sclerotium rolfsii Sacc. (Teleomorph : *Athelia rolfsii* (Curzi) Tu and Kimbrough) is a destructive fungal plant pathogen causing diseases in many mono and dicotyledonous plants encompassing more than 500 host species (Punja and Jenkins, 1984; Aycock, 1966). A number of extracelluar enzymes are produced adaptively by *S. rolfsii* these include pectin methylesterase (Bateman and Beer, 1965), cutinase (Baker and Bateman 1978), phosphatidase (Scallamn *et al.,* 1982, Tseng and Bateman, 1969) arabanase (Cole and Bateman, 1969), galactanase mannase and xylanase (Sadana *et al.,* 1980; Van Etten and Bateman, 1969) and β-glucosidase (Sadana *et al.,* 1983. Schewale and Sadana, 1981). Cellulase produced by the pathogen may play a secondary role in tissue destruction and disease development since they appear later in the temporal sequence of enzyme production (Punja and Grogan, 1981). Fungal hyphae grow through tissue both inter and intracellularly. They contain numerous microbodies (Hanssler *et al.,* 1978), the presumed site of oxalate synthesis (Armentroot *et al.* 1978) in addition to vacuoles that contain phosphatase. (Hansslet *et al.,* 1975). Pectic substances in cell walls are depleted in advance of growing hyphae (Smith *et al.,* 1984) and peroxidase is release (Barnett, 1974) through enzymatic action. The end result is the typical water-soaked, necrotic and macerated appearance of tissues infected by *S. rolfsii.* The importance of these enzymes in pathogenesis has not been conclusively studied. In this present investigation, enzyme production of different Isolates of *S. rolfsii in vitro* as well as how the fungus behaves in respect of enzyme production within the host tissue after inoculation have been undertaken.

MATERIALS AND METHODS

Samples were collected from different sources as well as different places followed by isolation and

confirmation of *Sclerotium rolfsii* Sacc. in 10 different Isolates namely 1 (from soil), 2 (from chilli), 3 (from elephant foot yam), 4 (from brinjal), 5 (from tuberose), 6 (from potato), 7 (from barley), 8 (from tomato), 9 (from black cumin), 10 (from groundnut), respectively. These Isolates were further assessed for their *in vitro* and *in vivo* enzyme activity.

For *in vitro* studies, all the isolates were grown in 250 ml Erlenmeyer flasks containing 50 ml Czapek's Dox Broth fortified with pectin (Sunskist Growmers Inc., Calif, USA) for pectic enzymes and carboxyl methyl cellulose sodium salt (CMC-Na) (BDH) for cellulase. The flasks were inoculated with 6 mm mycelial discs cut with a sterile disc cutter from the growing margins of 7th day old cultures on Potato Dextrose Agar medium and incubated at 28 ± 1°C. After requisite period of growth, the culture medium was filtered through folds of cheese cloth. The filtrate was centrifuged at 5000 rpm at 5°C for 15 min in a model PR-2 International Refrigerated Centrifuge. The decant was dialysed against several liters of distilled water for 20-24 hr at 5°C. The dialysate was used immediately as enzyme source.

For *in vivo* studies healthy groundnut seedlings were inoculated with isolate 10 respectively. Infected stem tissues were collected in three different Stages namely (Stage-1 i.e., 3 days after inoculation), (Stage-II i.e., 5 days after inoculation), and (Stage-III i.e., 7 days after inoculation). Collected stem tissues were washed with sterile distilled water and blended throughly in pre-chilled glass vessels in appropriate buffer of requisite pH in the ratio of 1:1 w/v. The blending and freezing was repeated thrice, the mass filtered through folds of cheese cloth, the filtrate centrifuged and supernatant collected and dialysed against several liters of distilled water as above. The dialysate was used immediately as enzyme source. Suitable checks were also maintained with un-inoculated stem.

For assay of enzyme buffers were prepared as described by Gomori (1955). The final pH was adjusted using Systronics photoelectric PH meter. Analar (BDH) or GR (E. Merck) chemicals were used for preparation of buffers. The buffer solution was preserved at 5°C for future use.

In vitro viscosimetric assay of pectinase and Cx was done following the method described by Bell *et al.* (1959). One ml enzyme was added to 9 ml buffered substrate in an Ostwald's viscosimeter and the flow time was recorded at specific time intervals. The enzyme susbtrate mixture was incubated in a thermostatic water bath at 30± 1°C. The flow time of water and flow time of bufferised substrate and dead enzyme (autoclaved for 10 min at 121°C) mixture (9:1) was also determined. The per cent reduction in viscosity was calculated from the formula : $PDFT_1 = (E - E_t) / (E - E_w) \times 100$; E = Flow time of substrate and dead enzyme; Et = Flow time of substrate + active enzyme preparation after time t; E_w = Flow time of distilled water.

Per cent decrease in flow time (PDFT) was plotted against incubation time and the time required for 50% reduction in visocosity was calculated. Specific enzyme activity where possible, was calculated as 1/t ×1000. Where, t is the time required in minutes for 50% reduction in viscosity by an enzyme preparation.

For viscosimetric assay of PG and Cx *in vivo,* 2 ml

The following buffers were used :

Name of buffer	Constituents	Stock molarity	pH range	Final molarity
Citrate	Citric acid and sodium citrate	0.1	3.0-6.2	0.01
Acetate	Acetic acid and sodium acetate	0.2	3.6-5.6	0.02
Citrate phosphate	Citric acid and dibasic sodium phosphate	0.1 and 0.2	3.2-7.0	0.02
Phosphate	Monobasic sodium phosphate and dibasic sodium phosphate	0.2	5.7-8.0	0.02
Tris aminomethane	Tris (hydroxy-methyl) aminomethane and hydrochloric acid	0.2	7.2-9.0	0.02

of enzyme preparation, 1 ml of 0.001M calcium chloride were added to 8 ml of bufferised substrate in an Ostwald's viscosimeter. Rest of the procedure was same as described for *in vitro* viscosimetric assay.

RESULTS AND DISCUSSION

Observations on enzyme activity of different isolates proved diversity among different isolates of *S. folfsii*. Highest activity of enzyme "cellulase" *in vitro* was observed in Isolate 6 and 8 (333.3) followed by Isolate 10 (322.5), Isolate 5 (285.0), Isolate 7 (256.4), Isolate 1 and 9 (250.0), Isolate 2 (243.9), Isolate 3 (117.6) and Isolate 4 (166.6) respectively (Table 1).

Results revealed variability and high cellulase activity by different isolates of *S. rolfsii*. High amount of cellulase activity by *S. rolfsii* can be related with the previous works done by various workers like Linder *et al.*, (1983) and Schewale and Sadana, (1979) who also reported significant cellulase production by this pathogen. The present study also revealed significant amount of cellulase production by different isolates of *S. rolfsii*. Apart from these it was also recorded that variability in respect of production of cellulase, varied from 117.6 to 333.3.

Highest activity of enzyme "pectinase" *in vitro* was observed in Isolate 5 (50.0) followed by Isolate 3 (18.18) (Table 1). For other isolates the PDFT 50 values could not be attained within one hour of incubation. But the PDFT t value of these isolates ranged between 28.0 to 48.0. Bateman and Beer (1965) also reported a number of extracellular enzyme of *S. rolfsii* including pectin methyl esterase and polygalacturonase in liquid culture media and in infected bean hypocotyls during pathogenesis. Bateman (1972) also revealed the production of polygalacturonase complex by *S. rolfsii*. Significant amoung of pectinase production by different isolates of *S. rolfsii* was established in our observations as well as variability in the production of pectinase was

Table 1 : *In vitro* enzyme activity of different isolates of *S. rolfsii* Sacc.

Readings	Activity of Cellulase *in vitro* (60 min)									
	Isolates									
		1	2	3	4	5	6	7	8	9 10
$PDFT_t$ after (60 min)	86	84	81	91	89	96	84	94	85	92
$PDFT_{50}$ (min)	4	4.1	8.5	6	3.5	3	3.9	3	4	3.1
Activity	250	243.9	117.6	166.6	285	333.3	256.4	333.3	250	322.5
	Activity of Pectinase *in vitro* (60 min)									
	Isolates									
	1	2	3	4	5	6	7	8	9	10
$PDFT_1$ after (60 min)	28	47	50	36	52	48	37	36	32	38
$PDFT_{50}$ (min)	-	-	55	-	20	-	-	-	-	-
Activity	-	-	18.18	-	50	-	-	-	-	-

Table 2 : Activity of cellulase and pectinase produced by *S. rolfsii* Sacc. Isolate (10) of groundnut *in vivo* (60 min)

	Activity of cellulase *in vivo*			Activity of pectinase *in vivo*		
Readings	$PDFT_t$	$PDFT_{50}$	Activity	$PDFT_1$	$PDFT_{50}$	Activity
Stage-I	79	4.3	232.0	69	15	66.66
Stage-II	85	3.9	256.41	59	17.3	57.80
Stage-III	82	4.0	250	51	20.1	49.75

also observed which varied from 18.18 to 50.0 after one hour of incubation of enzyme + substrate butter complex (Table 1).

In vivo studies revealed that the activity of cellulase increased from Stage I to Stage II and there after decreased slightly at Stage III. Thus we can conclude that activity of cellulase increased as the rotting progress from yellowing of leaves to drooping and wilting of plant, thereafter in decreased to some extent during complete collapse of plant. But in case of pectic enzymes it decreased from Stage I to Stage II and from Stage II to Stage III that is activity of enzyme was highest during yellowing of leaves (first visible symptom of disease) then it decreased till final stage that is up to collapse of the plant (Table 2). Cellulase produced by the pathogen may play a secondary role in tissue destruction and disease development since they appear later in the temporal sequence of enzyme production (Punja and Grogan, 1981). Fungal hyphae grow through tissue both intercellulary and intracellularly. Pectic susbtances in cell walls are deplected in advance of growing hyphae (Smith *et al.,* 1984) and peroxidase is released through enzymatic action. The end result is the water soaked necrotic and macerated appearance of tissues infected by *S. rolfsii.*

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