Volume 1, Issue 5 December 2015 ISSN NO: 2454 - 7514

North Asian International Research Journal Consortium

North Asian International Research Journal

Of

Science, Engineering and Information Technology

Welcome to NAIRJC

ISSN NO: 2454 -7514

North Asian International Research Journal of Science, Engineering & Information Technology is a research journal, published monthly in English, Hindi, Urdu all research papers submitted to the journal will be double-blind peer reviewed referred by members of the editorial board. Readers will include investigator in Universities, Research Institutes Government and Industry with research interest in the general subjects

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Address: - Dr. Ashak Hussain Malik House No. 221 Gangoo, Pulwama, Jammu and Kashmir, India - 192301, Cell: 09086405302, 09906662570, Ph. No: 01933-212815,
Email: nairjc5@gmail.com, nairjc@nairjc.com, info@nairjc.com Website: www.nairjc.com

QUANTITATIVE ESTIMATION OF SOME ENZYMES IN *SYZYGIUM CUMINI* (L.) SKEELS AFFECTED BY *CASSYTHA FILIFORMIS* (L)

VENU GOPAL A[1], RADHAIAH A[2], ARUNA KUMARI M[3] AND NAGALAKSHMI DEVAMMA M[4*]

1, 4* Department of Botany, Sri Venkateswara University, Tirupati.

2. Department of Botany, S.V.A. Govt. Degree College for men Sri Kalahasti, Chitoor Dist. A. P, INDIA.

3. Department of Botany Govt. Degree College Puttur, Chitoor Dist. A.P, INDIA.

ABSTRACT

This paper reports the quantitative estimation of some enzymes in affected *S.cumini* leaf. The investigation assayed were protease, RNase, Catalase, peroxidase**,** polyphenol oxidase, β-amylase, phenylalanine ammonia-lyase, glutamic dehydrogenase and ascorbic acid oxidase compared to healthy tissues, affected leaf showed significantly higher content of enzymes activity than healthy tissues.

KEY WORDS; - protease, RNase, Catalase, peroxidase**,** polyphenol oxidase, β-amylase, *S. Cumini*, *C. filiformis.*

INTRODUCTION

Syzygium cumini is a fast grown medicinally important plant which is originally being grown in forest lands of Indonesia and India. This tree belongs to the family Myrtaceae. All parts of these plants are medicinally useful and it has a long tradition in alternative medicines. The leaves and bark are used for controlling blood pressure and gingivitis. Wine and vinegar are also made from the fruit. It has a high source of vitamin A and vitamin C (Luximon-Rammal *et al.*, 2010) the ash of the leaves is used for strengthening the teeth and gums, leaves may be used as fodder**.**

Higher plant parasite (*Cassytha filiformis*) attack on *S. cumini* resulting wilting, reduction in plant size, loss of yield or low yield, lower quality, physiological changes in the host plant, **higher plant parasite** cause great damage to the plant or tree. The effect of infection on some of the enzymes like protease, ribonuclease, catalase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, β-amylase, glutamic dehydrogenase and ascorbic acid oxidase were studied.

MATERIAL AND METHODS

Assay of enzymes analysis of the Host – Parasite complex

Host-Parasite complex was analyzed using infected plant leaf extract / filtrate extract for various assay of enzymes viz, protease, RNase, Catalase, peroxidase**,** polyphenol oxidase, β-amylase, phenylalanine ammonia-lyase, glutamic dehydrogenase and ascorbic acid oxidase using standard procedures.

Enzyme assays

The enzymes such as protease, RNase, Catalase, peroxidase**,** polyphenol oxidase, β-amylase, phenylalanine ammonia-lyase, glutamic dehydrogenase and ascorbic acid oxidase were determined quantitatively in both healthy and affected leaves of *S. cumini.*

1. Protease activity

Protease activity was estimated by the method of Kunihiko Naito *et al.,* (1979) where one gram of leaf sample was homogenised with 5 ml of ice cold 0.1M phosphate buffer (pH 6.5). The homogenate was centrifuged at 11,500 g for 30 min and the supernatant was assayed. Proteolytic activity was determined as the capacity to hydrolyze casein. In the assay, 1 ml of enzyme extract was incubated with 2 ml of 1% vitamin free casein solution in sodium phosphate buffer (pH 6.5) and incubated at 37°C for 2 hr. Then, 1 ml of cold TCA was added and the mixture was centrifuged. Proteolytic activity was measured by absorbance at 750 nm of the TCA soluble fraction (supernatant) after reacting with Folin-phenol. Reagent against a zero time blank was taken according to the method of Lowry *et al.,* (1951). Enzyme activity was measured in arbitrary units as one enzyme unit, defined as an increase of one A_{750} unit per hour under the assay conditions.

2. Ribonuclease activity

RNase activity in the leaf samples were determined quantitatively according to the method of Bagi and Farkas (1967) where one gram of sample was homogenized in a prechilled mortar at 2-4°C with distilled water. The homogenate was squeezed through a four layered muslin cloth and the extracts were centrifuged at 3000 rpm for 15 minutes in an MSE refrigerated centrifuge. The supernatant thus obtained was used to assay for RNase activity.

The assay system containing 0.5 ml enzyme extract, 2 ml of 7.5 mg/m1 yeast RNA and 0.5m1 0.IM acetate buffer (pH 5.6) was incubated at 37°C in a water bath for 30 min, terminated by adding 0.5ml of Mc Fadyens reagent (0.25% Uranyl acetate is 2.5% TCA), shaken well and set overnight at 4°C. The mixture was centrifuged and the supernatant diluted (1/10) with distilled water. O.D. values were measured against a zero time control at 260 nm with a Schimadzu UV-240 spectronic spectrophoto-meter. Activity was measured in arbitrary units/ml as

$$A_{260} = \frac{\text{(ml assay solution + ml precipitating agent) dilution factor}}{\text{ml enzyme solution assayed} \times \text{time in minute}}$$

3 .Catalase activity

Catalase enzyme activity was measured according to the method of Gopalachari (1963) where 200mg of leaf material was ground with 20ml of cold phosphate buffer (p^H 7.0) and the homogenate was filtered through pyrex glass wool and then made up to 25 ml. The reaction mixture (2 ml enzyme extract and 1.0 ml of 0.045M H_2O_2) was incubated for 5 min and the reaction stopped by adding 1.0 ml of 12% sulphuric acid. The mixture was titrated immediately against 0.05 N $KMn0_4$ and the end point was denoted by the first appearance of a pink

colour, which lasted for 30 seconds. The blank was run simultaneously substituting the enzyme extract by an equal volume of buffer. Enzyme activity was expressed as mg of H_20_2 destroyed in 15 minutes per gram weight of the tissue.

C = 25/2 x 0.85 x V/W

V = Difference in titre value between control and treated

W = Weight of the sample in grams.

0.85µg H_2O_2 = 1.0 mg $KMnO_4$

4. Peroxidase and poly phenol oxidase activity

Peroxidases activity was estimated according to the method of Manoranjankar and Dinabandhu (1976) by grinding the leaf sample with 0.1 M phosphate buffer (pH 7.0) in a pre-chilled mortar and centrifuging the homogenate at 15000 g at 4^{o}C for 30 min. the aliquot obtained was used as the source of the enzyme.

The assay mixture for peroxidases contained 2 ml 0.1M phosphate buffer (pH 7.0), 1ml 0.01 M pyrogallol, 1 ml 0.005 M H_2O_2 and 1 ml enzyme extract. The reaction was stopped by adding 1 ml of 2.5 N sulphuric acid after 5 min incubated at 25^{o}C and the amount of peroxidases formed was estimated by measuring absorbance at 420 nm enzyme activity was recorded in terms of absorbancy units.

The method followed to estimate polyphenol oxidase activity was same as that for peroxidases except that the reaction mixture contained no H_2O_2. Absorbance was measured at 420 nm.

5. β-amylase activity

β-amylase activity was estimated according to the method of Bernfeld (1955) where an aqueous extract of the material was prepared by grinding one gram of leaf tissue in 10 ml chilled water. The extract was filtered and the filtrate was made up to 10 ml. A one ml sample of the extract was incubated for three minutes at 20°C with 1 ml of starch solution (1g of starch dissolved in 100 ml of 0.016 M acetate buffer, pH 4.8). The enzyme reaction was then interrupted by the addition of 2 ml of 3,5-dinitro salicylic acid reagent (1 g of 3, 5-DNS in 20 ml of 2 N sodium hydroxide and 50 ml distilled water; 30 g of Rochelle salt was added and the mixture made up to 100 ml with distilled water). The tube was heated for 5 min in a boiling water bath and cooled in running tap water. After adding 20 ml water, the optical density of the solution containing a brown reduction product was determined photo metrically at 540 nm. A blank was maintained without adding the enzyme. A calibration curve established with maltose was used to convert the data into mg of maltose. Amylase activity was measured in terms of mg of maltose liberated in 3 min at 20°C by 1 ml of the enzyme solution.

6. Phenylalanine ammonia lyase

Activity of PAL-ase was determined according to the method of Biehn *et al*, (1968) with slight modification as follows:

One gram of freshly harvested material was ground in a chilled mortar with 10 ml of ice cold 0.1M borate buffer, pH 8.8 at 4-5°C, in a cold room. The homogenate was filtered through several layers of muslin cloth. The extract was then centrifuged in the cold at 10000 g for 30 min. The supernatant was made up to 15 ml and used to measure PAL-ase activity.

The reaction mixture (2 ml of enzyme extract, 1 ml of 0.05M L-Phenylalanine and 2 ml of 0.1M Borate buffer pH 8.8) was incubated for 1 hr in a *test* tube at 40°C and the reaction was stopped by adding 0.1 ml of 5 N HCl. A zero time control prepared at the same time was assayed. The acidified reaction mixture was measured at 268 nm in Rilger and Watts spectrophotometer. The values, in terms of cinnamic acid formed, were calculated from a standard curve prepared from known amounts of cinnamic acid.

7. Glutamic Dehydrogenase Activity

GDH activity was determined by the procedure of Smith and Way good (1963) where one gram of freshly harvested leaf material was ground at 0°C in a chilled mortar with 10.0 ml of ice cold phosphate buffer, pH 8.0, with 0.01 M cysteine (1.2 w/v) and filtered. The filtrate was centrifused at 20000 rpm for 30 min at 0.2^{o}C. The supernatant was made up to 20 ml and used as a source of enzyme.

The enzyme was assayed at 21± 1°C following the oxidation of NADH at 340 nm in a spectrophotometer using α-ketoglutarate and ammonia as substrates. To the reaction mixture (0.2 ml of NADH (0.2 µ moles), 0.5m1 $(NH_4)_2SO_4$ (750µ moles), 1.9 ml 0.1M phosphate buffer (pH 8.0) and 0.2 ml enzyme extract), 0.2 ml α-ketoglutarate (40 µ moles) was added. Optical density readings were made at 1 minute intervals and the specific activity was calculated as

EV/mg protein

Where

E = Extinction decrement during a six minute period.

V = Volume of the reaction mixture.

8 Ascorbic acid Oxidase

Five hundred mg of fresh leaf material was ground at 0°C in a chilled glass mortar with 20 ml of ice cold phosphate citrate buffer, pH 5.0. The homogenate was filtered through pyrex glass wool and made up to 25 ml with the same buffer and used for the enzyme assay. The reaction mixture (4.0 ml of enzyme assay, 2.5 ml of 2% ascorbic acid reagent) in 25 ml conical flasks was incubated for 30 min and the reaction was stopped by the addition of 1 ml of 1% TCA. For the control, 1ml of TCA was added to the reaction mixture containing 4 ml of ascorbic acid reagent without any time lag. The total volume in each of the flask was made up to 10ml. One ml of this solution was titrated against 0.001N 2,6-DCP (30 mg of 2, 6 dichlorophenol dissolved in 100 ml of distilled water to which two or three drops of 0.1N NaOH was added) until a permanent pink colour was attained. Ascorbic acid oxidase activity was measured for 30 min and recorded per gram fresh weight of tissue. The specific enzyme activity was determined as

A=V/W x 0.008 x 10 x 25/4

Where

A=ascorbic acid oxidase activity expressed as mg of ascorbic acid per gram fresh weight of plant tissue.

V=difference in titre values between the control and treatment in ml

W=fresh weight in gram of plant material 0.008 mg ascorbic acid =1ml of 2, 6–DCP.

RESULTS

Assay of enzymes analysis of the Host – Parasite complex

The effect of infection was some of the quantitative changes in enzymes like protease, ribonuclease, catalase, peroxidase, polyphenol oxidase; phenylalanine ammonia lyase, β-amylase, glutamic dehydrogenase and ascorbic acid oxidase were studied.

All the results were computed on gm fresh weight basis and changes in various constituents due to infection expressed as percent increase (marked by '+' sign) or decrease (marked by '–'sign) over healthy plants at different seasons of infection / disease development.

Enzyme assays

Protease enzyme activity: It was higher in affected leaves than that of healthy leaves of *S.cumini* (Table-1 and Fig-1). In the affected plants the activity was greater by 7.5%, 6.4% and 21.3% at different seasons respectively.

Ribonuclease enzyme activity: The activity of Ribonuclease enzyme showed fluctuations with high and low values in both healthy and affected plants. It was higher in affected leaves of *S. cumini* than unaffected plant samples (Table-2and Fig-2). In the affected plants the activity was greater by 188.8%, 200.0% and 126.7% at monsoon, summer and winter seasons respectively.

Catalase enzyme activity: Higher activity of the enzyme catalase was quantified in the case of affected plant samples. In infected host samples the activity was high by 16.93%, 25.26% and 18.53% at different seasons respectively. In this plant the activity was higher at monsoon and winter and lower at summer season (Table-3 and Fig-3).

Peroxidase enzyme activity: It was higher in infected leaves than that of healthy leaves. In the inoculated plants the activity was greater by 15.13%, 9.72% and 11.50% at different seasons respectively when compared to the corresponding healthy samples (Table-4 and Fig-4).

Polyphenol oxidase enzyme activity: The activity of polyphenol oxidase enzyme was high by 17.93%, 17.36% and 7.17% at different seasons summer, monsoon and winter respectively, in the affected leaves of *S. cumini* as comparison with the corresponding healthy samples. In the sample the activity was high during summer, monsoon and low in winter season (Table-5 and Fig-5).

Table-1: Protease enzyme activity in healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection (expressed in arbitrary units per hour gram fresh weight of leaf)*

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	0.163 ± 0.001	0.175 ± 0.003	7.566
Monsoon	0.124 ± 0.002	0.132 ± 0.002	6.451
Winter	0.137 ± 0.01	0.167 ± 0.001	21.307

*Average values of triplicate samples

POC – Percent Over Control

± Standard deviation

Table – 2: Ribonuclease enzyme activity (units / ml)* of healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	26.43 ± 0.01	76.35 ± 0.03	188.84
Monsoon	21.27 ± 0.01	63.84 ± 0.02	200.04
Winter	23.75 ± 0.01	53.74 ± 0.02	126.27

*Average values of triplicate samples

POC – Percent over Control

± Standard deviation

Table-3: Changes in catalase activity (mg/ g fr.wt)* in healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	0.183 ± 0.02	0.214 ± 0.002	16.93
Monsoon	0.147 ± 0.01	0.182 ± 0.002	23.26
Winter	0.163 ± 0.01	0.194 ± 0.001	18.53

*Average values of triplicate samples

POC – Percent Over Control

± Standard deviation

Table – 4: Peroxidase activity (specific activity per mg protein)* in healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection.

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	1.695 ± 0.003	1.955 ± 0.01	15.133
Monsoon	1.637 ± 0.001	1.797 ± 0.01	9.729
Winter	1.663 ± 0.01	1.854 ± 0.001	11.505

*Average values of triplicate samples

POC – Percent over Control

± Standard deviation

Table-5: Polyphenyl oxidase activity in healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	1.634 ± 0.02	1.927 ± 0.001	17.93
Monsoon	1.588 ± 0.01	1.863 ± 0.01	17.36
Winter	1.534 ± 0.002	1.644 ± 0.002	7.17

*Average values of triplicate samples
POC – Percent Over Control
± Standard deviation

Table-6:Phenylalamineammonialyase activity (expressed as cinnamic acid formed in mg/ g fr.wt)* in healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	21.54 ± 0.01	29.32 ± 0.02	36.1
Monsoon	17.24 ± 0.01	19.66 ± 0.01	14.08
Winter	21.36 ± 0.01	24.34 ± 0.02	13.93

*Average values of triplicate samples
POC – Percent Over Control
± Standard deviation

Table-7: β-amylase enzyme activity (mg maltose produced / ml aquous extract)* in healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection.

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	2.576 ± 0.01	3.86 ± 0.02	49.806
Monsoon	2.176 ± 0.01	3.14 ± 0.01	44.257
Winter	2.36 ± 0.02	3.46 ± 0.02	46.61

*Average values of triplicate samples
POC – Percent Over Control
± Standard deviation

Table-8; Glutamic dehydrogenase activity (specific activity / mg protein)* in healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	0.257 ± 0.015	0.277 ± 0.002	7.503
Monsoon	0.185 ± 0.003	0.257 ± 0.001	39.03
Winter	0.216 ± 0.002	0.265 ± 0.001	22.69

*Average values of triplicate samples
POC – Percent Over Control
± Standard deviation.

Table – 9: Ascorbic acid oxidase activity of healthy and *C. filiformis* affected *S. cumini* leaves at different seasons of infection.

Seasons	*S. cumini*		
	Healthy	Affected	POC
Summer	3.163 ± 0.01	3.84 ± 0.02	21.39
Monsoon	1.633 ± 0.01	2.543 ± 0.01	55.71
Winter	1.943 ± 0.02	3.19 ± 0.01	64.15

*Average values of triplicate samples
POC – Percent Over Control
±Standard deviation

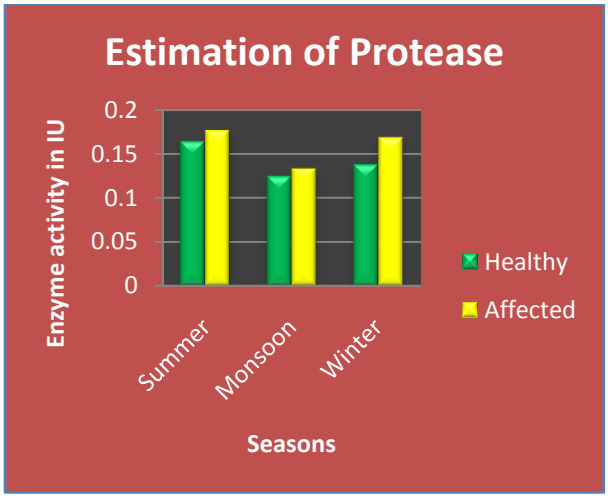

Fig-1: Bar diagrams showing the changes in Protease enzyme activity of healthy and affected *S. cumini* leaves

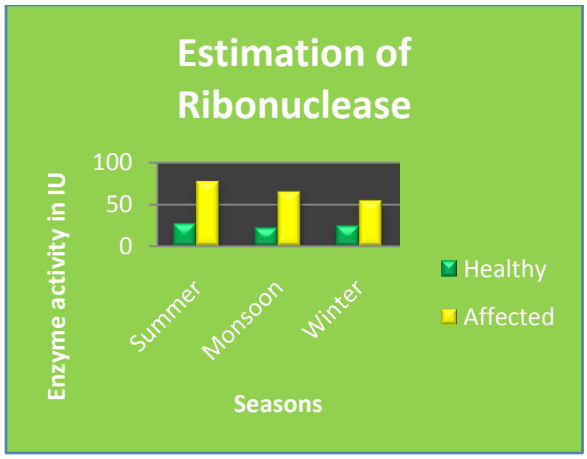

Fig-2: Bar diagrams showing the changes in Ribonuclease enzyme activity of healthy and affected *S. cumini* leaves.

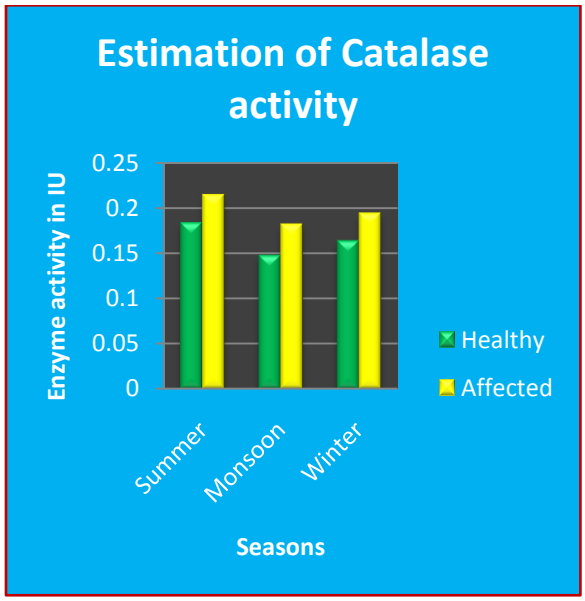

Fig-3: Bar diagrams showing the changes in catalase enzyme activity of healthy and affected *S. cumini* leaves.

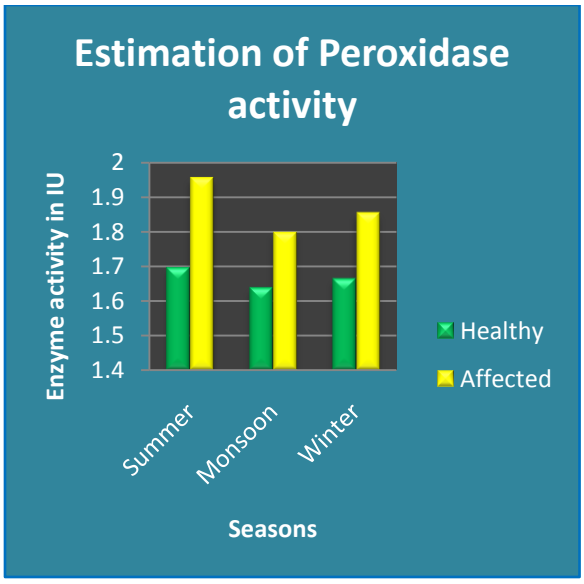

Fig-4: Bar diagrams showing the changes in peroxidase enzyme activity of healthy and affected *S. cumini* leaves.

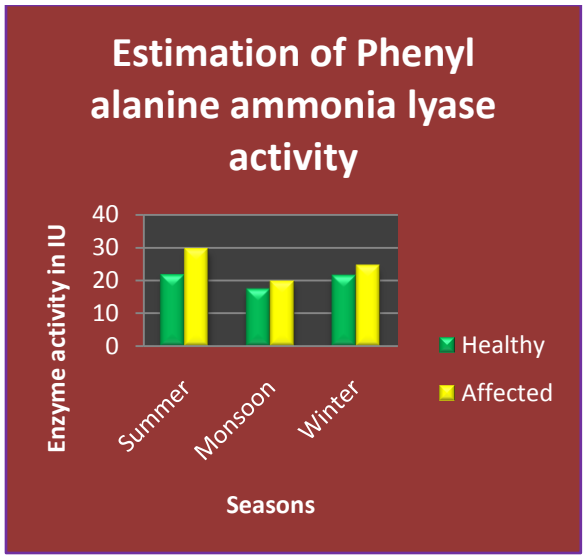

Fig-6: Bar diagrams showing the changes in Phenylalanine ammonia lyase enzyme activity of healthy and affected *S. cumini* leaves

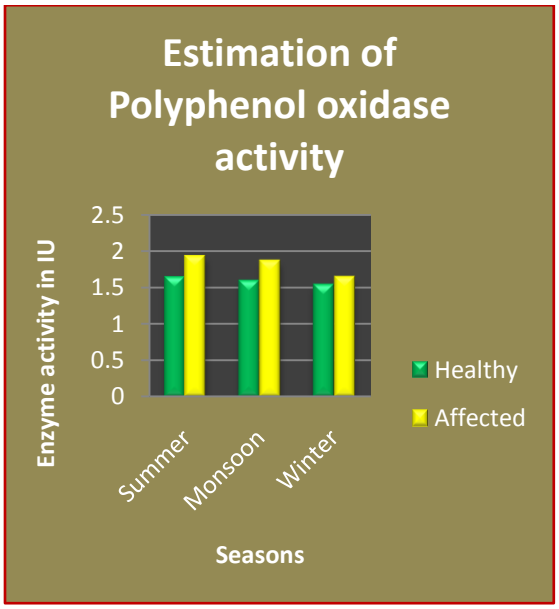

Fig-5: Bar diagrams showing the changes in Polyphenyl oxidase enzyme activity of healthy and affected *S. cumini* leaves.

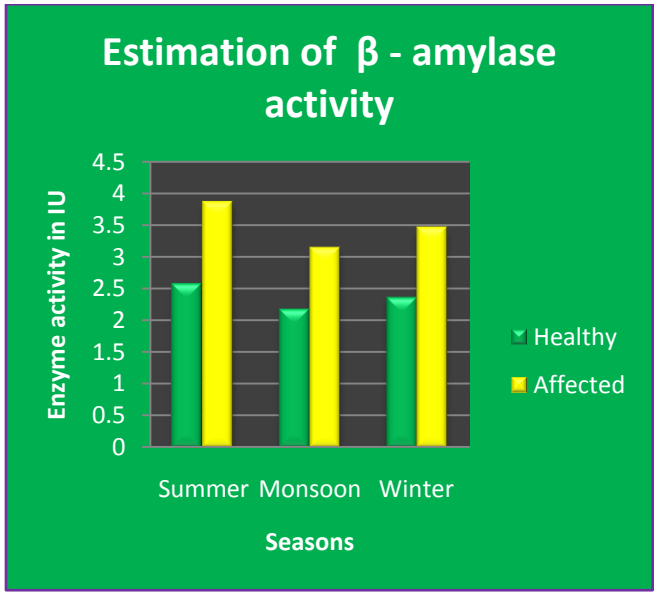

Fig-7: Bar diagrams showing the changes in β-amylase enzyme activity (mg maltose produced / ml aquous extract)* of healthy and affected *S. cumini* leaves.

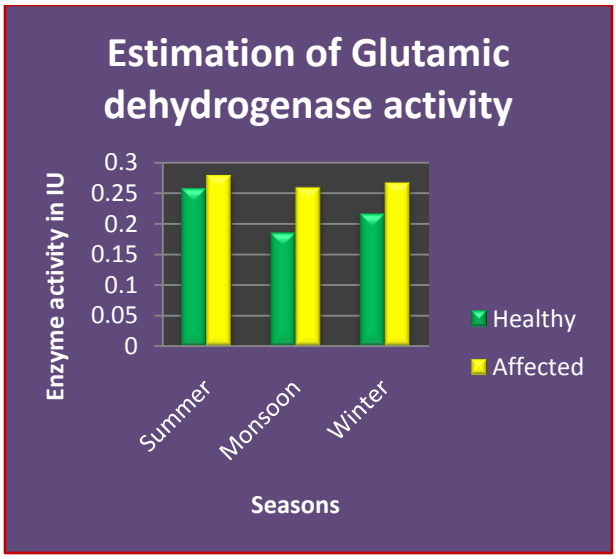

Fig-8: Bar diagrams showing the changes in Glutamic dehydrogenase activity (specific activity / mg protein)* of healthy and affected *S. cumini* leaves.

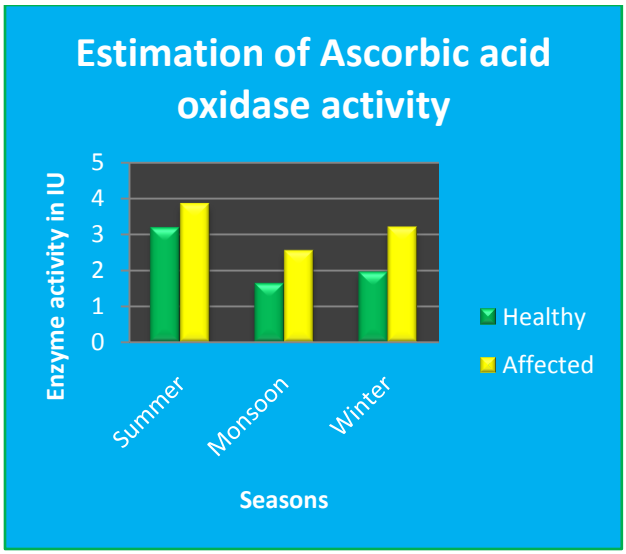

Fig-9: Bar diagrams showing the changes in Ascorbic acid oxidase activity of healthy and affected *S. cumini* leaves

Phenylalanine ammonia lyase activity: The activity of Phenylalanine ammonia lyase showed fluctuations in both healthy and affected leaves. The activity was higher in affected leaves than that of healthy *S. cumini* leaves. The activity was greater by 36.1%, 14.08% and 13.93% at different seasons respectively, when compared to corresponding healthy samples (Table-6 and Fig-6).

β-amylase activity: It was higher in the affected *S. cumini* plant (Table-7 and Fig-7). The enzyme activity in affected leaves was greater by 49.80%, 44.25%, and 46.61% at different seasons summer, monsoon, winter respectively, when compared to corresponding healthy samples.

Glutamic dehydrogenase (GDH) activity: The GDH activity in the leaves of affected plants was higher than that of the healthy tissues (Table-8 and Fig-8). In the affected plants the activity was greater by 7.5%, 39.0% and 22.69% at different seasons respectively.

Ascorbic acid oxidase (AAO) activity: It was higher in affected leaves over healthy leaves of *S. cumini*. The enzyme activity in affected leaves was greater by 21.39%, 55.71% and 64.15% at different seasons summer, winter, monsoon respectively, compared to the corresponding healthy samples (Table-9 and Fig-9). The AAO activity increased with aging in the *S. cumini* i.e, high in monsoon and winter and lower in summer.

DISCUSSION

The aspect of this paper is on host – parasite physiology of *S. cumini* and *C. filiformis*. Photosynthetic activity and nutrients like carbohydartes, which are the photoassimilate substances, are very important in terms of parameters that explain the physiological activity of the plants (Semra, 2004). For this we have analyzed the enzyme assays.

In the present study, enzymological altrations in infected leaves of the host *S. cumini* by *C.filiformis* was compared with healthy host leaves. Significant differences were observed in all the enzyme assays parameters.

Increase in enzyme activities is a general phenomenon in infected tissues it may be due to the synthesis of enzymes and proteins induced by the pathogen or due to the activation of inactive enzymes (Shaw, 1963). In the present studies a considerable increase in the peroxidases, polyphenoloxidase, PALase, β- amylase, GDH ase, AAOxidase activities. The amount of glutamate dehydrogenase, and protein in leaves of infected plants increased compared with controls (Hibberd *et al*., 1998).

More than one mechanism increased synthesis in the host (Goodman *et al.*, 1967); activation of its latent enzymes (Farkas *et al.*, 1964) or direct synthesis by the parasite (Farkas and Kiraly, 1962) is ascribed to be responsible for the activation of these phenol oxidizing enzymes in the infected tissues. Alterations in the enzymatic activities due to infection were also quite evident. The activities of peroxidase, polyphenol oxidase, catalase, β-amylase, Ascorbic acid oxidase, Glutamic dehydrogenase, Protease, R Nase, phenylalanine ammonialyase increased to varying degrees at various seasons of disease development.

The main objective of the present investigation has been to understand the host-parasite interaction between *S. cumini* and *C. filiformis* the study broadly covers host – parasite physiology of *S.cumini* - *C.filiformis* in terms of enzymological characteristics of the host parasite interaction / complex.

SUMMARY AND CONCLUSION

Overall consideration of the results of the affected plants it may be concluded that *C. filiformis* interferes with various biochemical and enzyme biosynthesis mechanisms of the host plant for the benefit of the parasite growth and reproduction, thus reducing the yield potentials of the host plant.

From the above results of the investigation it was clear that the parasite *C. filiformis* continuously absorbing the host plant's nutrients and metabolite The high quantities of enzymes in the infected plants in turn indicate the defense developments.

ACKNOWLEGMENT; - *Authors wish to thank University Grant Commission, Govt. of India. New Delhi for financial support through BSR Fellowship to carry out the present research work.*

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Address:- Dr. Ashak Hussain Malik House No-221, Gangoo Pulwama - 192301 Jammu & Kashmir, India
Cell: 09086405302, 09906662570,
Ph No: 01933212815
Email:- nairjc5@gmail.com, nairjc@nairjc.com , info@nairjc.com
Website: www.nairjc.com